Importance of fatty-acyl composition in production and reconstitution of active dried Saccharomyces cerevisiae.

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IMPORTANCE OF FATTY-ACYL COMPOSITION IN PRODUCTION AND RECONSTITUTION OF ACTIVE DRIED *Saccharomyces cerevisiae*

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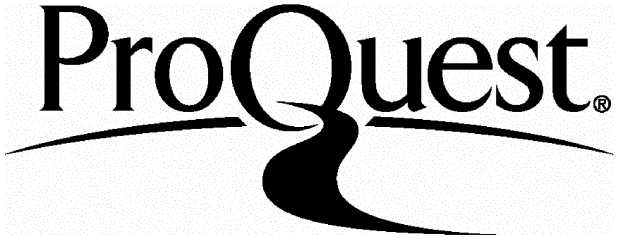

SUMMARY

A study was made of the importance of lipid composition, and particularly of the fatty-acyl composition of lipids, on the capacity of strains of Saccharomyces cerevisiae to retain fermentative power, when they were converted into an active dried form under conditions similar to those used on a commercial scale. Three strains of Saccharomyces cerevisiae were used. A strain isolated from commercial active dried yeast was designated the Parent strain. A mating strain obtained from an ascospore of this strain was also used and designated Mating Parent strain HP 92. Finally mutagenic treatment of the Mating Parent strain with N-Methyl-N'-Nitro-N-Nitroso-guanidine led to the isolation of a strain which is auxotrophic for unsaturated fatty acids. This was designated mating strain RP 108. Growth of the auxotrophic mutant strain RP 108 in aerobic batch-culture was promoted by supplementation of the medium with oleic acid ($C_{18:1}\Delta^{9}$ cis) in pure form or as Tween 80. A preliminary investigation of the specificity of fatty-acid requirement of this strain indicated that other unsaturated fatty acids (notably linoleic acid, $C_{18:2}\Delta^{9,12}$ all cis) of both different chain length and site and number of unsaturations could satisfy the growth-requirement. The requirement for an unsaturated fatty acid was not absolute, as some growth occurred in unsupplemented cultures.

All three strains were grown aerobically in batch-culture under conditions of restricted growth rate, and both nitrogen and phosphate limitation. Yeasts were harvested

and converted to active dried yeasts in a laboratory-scale tray drier. Following reconstitution in water at either 38 or 25°C the fermentative activies of the yeasts were compared with those of the yeasts prior to drying. Cultures of auxotrophic strain RP 108 were grown under various conditions of unsaturated fatty-acid supplementation. Recovery of fermentative activity from active dried yeasts prepared from such cultures was greatest when the medium had been supplemented with a high concentration of $C_{18:1}\ \Delta^9$ cis acid, and was comparable to that value obtained from cells of the Mating Parent strain.

Subsequent analyses of fatty-acyl residues from whole-cell, phospholipid and isolated plasma membrane preparations from both the Mating Parent and auxotrophic RP 108 strains indicated that fatty-acid supplements were incorporated by the latter strain in a chemically unmodified form, primarily into the phospholipids and only into neutral lipids when supplied in excess. A close correlation existed between the fatty-acyl compositions of phospholipids and plasma membranes isolated from crops of both strains. Cells of the auxotrophic strain RP 108, containing various proportions of unsaturated fatty acid, contained similar amounts of total lipid, free fatty acid, total phospholipids and free sterols. However, differences were observed in the contents of individual phospholipids, individual sterols and esterified sterols isolated from such cells.

The function of unsaturated fatty-acyl composition and membrane stability, particularly with regard to the stresses of drying and reconstitution in active dried yeast, is discussed.

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INTRODUCTION

Yeasts, particularly strains of Saccharomyces cerevisiae have for many years been popular organisms for studying biochemical processes. Being unicellular they are relatively easy to manipulate in the laboratory, both physiologically and genetically, yet the information gained from such manipulations may have direct relevance to higher, more specialised cells due to the eukaryotic nature of the yeast cell. In addition, yeasts are of considerable economic importance to man. They have, for many centuries, been responsible for one of man's staple diets; namely leavened bread. In recent times it has been economically desirable to produce yeast for the baking industry in a stable dry form, as active dried yeast (ADY). The production of ADY imposes considerable stress on the cell due to the removal of water during the drying process. The chemical and physical characteristics of the yeast plasma membrane are considered to be important, perhaps even of prime importance, in governing the resistance of the cell to the potentially lethal stress of drying.

The object of the present study was to examine the effects of alterations in cell lipid and plasma membrane fatty-acyl composition, on the resistance of yeast cells to the stresses of drying. In addition it was hoped that while such information might be beneficial to our understanding of the properties of ADY, it might also aid our general knowledge of the role of fatty-acyl

composition in membrane structure and function. In any such study the ideal approach is to effect specific changes in the chemical composition of the organelle being studied. This was attempted, with a high degree of success, by obtaining an unsaturated fatty-acid auxotrophic mutant strain of Saccharomyces cerevisiae, which was subsequently grown under various conditions of fatty-acid supplementation. By such means, cells and their plasma membranes, were enriched with one or more long-chain fatty acids. The degree of enrichment and the resistance of these cells to drying was then determined.

ACTIVE DRIED YEAST

Active dried yeast (ADY) is a preparation of viable cells of suitable yeast strains that have been dehydrated to a low moisture content (less than 10%, w/w) by one of several alternative drying methods. The strains used by the world's commercial bread-baking industries appear to be exclusively strains of Saccharomyces cerevisiae.

The use of ADY in the baking industry has several attractive advantages over the use of fresh yeast (often known as cake or compressed yeast) which has a water content within the range 65-75% (w/w). Firstly, due to a greatly diminished water content, ADY has a shelf life measured in months and is usually successfully stored at, or in tropical climates just

below, the ambient temperature. In contrast, fresh yeast requires refrigeration from the time of production to final use in the bakery, and even so it only has a shelf-life of two to three weeks. Secondly, the transportation costs for ADY are considerably lower than those for fresh yeast, because the requirement for refrigeration is eliminated and because the weight of the product at equivalent activity is decreased by up to 60%. Thirdly, ADY can be handled more easily within the bake-house, although it does require careful rehydration prior to use (Thorn and Reed, 1959). With these advantages over fresh yeast, ADY shows considerable potential not only in the home market but also for export to countries that do not possess the technology required for production or storage of fresh yeast. A large demand for ADY was precipitated by the outbreak of the Second World War when it was apparent that fresh yeast could not be conveniently used by the armed forces in the field. From some 1.4 million Kg in 1940, World production rose to over 6 million Kg in 1945, practically all being used by the armed forces. Although production initially dropped with the cessation of hostilities, ADY has been increasingly used by commercial bakeries since that time, particularly with the advent of packaged bread and roll mixes. Indeed, the recent unprecedented rise in the cost of energy for storage and transportation systems is likely to have ensured an ever increasing market for ADY.

Unfortunately, ADY also has several disadvantages when compared with fresh yeast. These disadvantages are outlined below, although several of the points will be discussed in greater detail later in this Introduction.

(1) Yeast for drying (often a strain chosen because of its resistance to drying) has to be grown under carefully controlled conditions different to those employed for the production of fresh yeast. In particular the total nitrogen and phosphate contents have to be maintained below certain predetermined values for the cells to resist the stresses of drying.

(2) Active dried yeast, after reconstitution in water, is less active on a dry weight basis when compared with fresh yeast. There are two reasons for this; firstly, the fermentative activity, before drying, of yeast cells grown under the restricted growth conditions already described, is lower than the fermentative activity of fresh yeast. Secondly, there is a further loss in viability and fermentative activity when the yeast is dried and then reconstituted, even under optimal conditions. As a consequence, the baker may have to add up to 50% (as dry wt.) extra ADY to the dough to obtain similar rates, and final levels of fermentation compared with doughs containing fresh yeast (Thorn and Reed, 1959; Šestáková, 1973b). The choice of reconstitution conditions, particularly that of temperature, is critical for the recovery of full

potential activity of any ADY preparation. Most workers in the field advocate a reconstitution temperature within the range 35 - 45°C to prevent loss of activity due to 'cold shock' effect first reported by Peppler and Rudert (1953).

(3) As a consequence of having to use more ADY than fresh yeast during the baking process, certain changes in flavour and colour of the bread (traditionally white in Great Britain) may occur which are not welcomed by the baker (Burrows and Fowell, 1968). Furthermore the presence of a large proportion of dead or less active cells results in an undesirable effect on the quality of the baked goods due to the poor volume and coarse nature of the crumb produced. This is due to the liberation of glutathione from the cells, which has a stimulating effect on the proteolytic enzymes of the yeast. (Shultz and Swift, 1955).

PREPARATION OF YEAST CELLS SUITABLE FOR DRYING

Strain Selection

Several workers have advocated the use of selected strains of *Sacch. cerevisiae* for the production of ADY (Merritt, 1957; Pyke, 1958; Čulina, 1969). These strains are either more resistant to the stresses of drying or show improved storage characteristics compared with strains used for the production of fresh yeast. More

recently the Koninklijke Nederlandsche Gist-en Spiritus-fabriek N.V. (1973)reported the use of certain hybrid strains that possessed high initial fermentative activities comparable to those obtained from some commercial yeast strains. Furthermore, the ADYs produced from the new hybrids recovered up to 91% of their initial fermentative activities before drying. Both of these factors resulted in a 45% improvement in recoverable fermentative activity of the ADY when the hybrid strains rather than the commercial ADY strains were used. Little information is available, however, to explain the biochemical factors responsible for the increased resistance to drying found in such strains.

The deleterious effects of glutathione from poor quality ADY on loaf volume and crumb texture have already been mentioned. Schultz and Swift (1955) showed how this effect could be minimized by producing a mutant strain of Sacch. cerevisiae that not only had a low glutathione content but could also metabolise that compound. Breads baked with ADY from the mutant strain were of comparable quality to those produced using fresh yeast, and a large improvement over the coarse breads baked with wild-type ADYs.

Growth Conditions

The principles involved in, and methods used for, the large-scale production of yeast cell have been

adequately reviewed by Burrows (1970), and Reed and Peppler (1973). The process most widely used is that known as the 'fed batch' or 'Zulauf' process. In this method carbohydrate (often molasses) is fed incrementally to the culture during the growth period, so that the sugar concentration, growth rate and chemical composition of the yeast cells are under external control. As a consequence, greatly improved cell yields and quality, particularly stability, can be obtained. Such results are not obtainable from either batch cultures or continuous cultures, and as yet continuous propagation systems present too many technological and microbiological problems to justify replacement of the fed-batch system. Yeast cells are grown aerobically in multi-vessel systems at a growth rate within the range 0.08-0.18 generations/h to ensure efficient nutrient utilisation. For a discussion of these aspects see Burrows (1970).

Propagation of yeast cells suitable for drying differs in some important aspects from those used for the production of fresh yeast (Oyaas et al.,1948). The total nitrogen content of the yeast, frequently used as a measure of the protein content, should be low and within the range 6-7% dry wt. equiv. (Frey, 1957; Thorn and Reed, 1959). This condition has been confirmed by Šestáková (1973b) who also stated that the total phosphate content (expressed as phosphorus pentoxide) of the yeast should not exceed 2.4% dry wt. equiv.

For the production of fresh yeast a 'ripening period' of 30-60 min is advocated after the cessation of nutrient feeds. This is believed to facilitate complete assimilation of nutrients, and to produce a degree of synchrony within the yeast population (Burrows, 1970). To achieve a similar effect in yeast cells produced for drying Schneider (1954) recommended the use of a higher temperature (36°C rather than 30-33°C) towards the end of the growth period, together with the addition of spent wash containing ethanol rather than fresh molasses. Synchronisation of the budding cycle by alternate feed and starvation periods may also be beneficial (Schuldt and Seeley, 1966).

The effects of these restricted growth conditions on yeast cell structure and biochemistry and their relevance to ADY are worth considering in further detail. Their effect on cell lipids and membranes will be considered later in this Introduction. Harrison and Trevelyan (1963) reported that, even if ADY cells were reconstituted at their optimal temperature, losses in fermentative activity could be severe or even total if the nitrogen content of the yeast had been allowed to exceed certain limits. One strain of yeast was found to lose only 15% of its activity after drying and reconstitution, provided that the organism had been grown with a total nitrogen content restricted to 6.4%. However, at 8.3% nitrogen, 40% of activity was lost and at 9.3% nitrogen, the suspension of yeast cells was

totally inactive. Similarly, Johnston (1963) found that whereas fresh baker's yeast ordinarily had a protein content in the range 47-52% and a phosphorus content in the range 2.6-4.0% (calculated as phosphorus pentoxide), the nitrogen and phosphorus contents of most yeasts used for drying were within the ranges 38-44% and 1.6-2.4% respectively.

The investigation of McMurrough and Rose (1967) is of interest in relation to the low nitrogen specification for ADY (Harrison and Trevelyan, 1963). In a study of the composition and structure of yeast cell walls isolated from Sacch. cerevisiae grown in continuous culture at different growth rates, under three conditions of glucose limitation and one condition of ammonium ion limitation, it was found that, (a) the glucose and mannose contents of the cell walls were relatively little affected by the growth rate; (b) the phosphorus and protein contents of the walls from yeast grown under each of the four conditions increased as the growth rate decreased; (c) walls from yeast grown under ammonium ion limitation contained only half as much protein as walls from cells grown under glucose limitation; (d) the proportion of lipid was greatest in walls from ammonium ion-limited cells; (e) the β-fructofuranosidase activity of the yeast grown under ammonium-ion limitation was much lower than that from yeast grown under conditions of glucose limitation. Furthermore, the walls of cells from ammonium-ion limited yeast were cylindrical, rigid

and had a much more spongy texture on the inside, whereas those from yeast grown under glucose limitations were oval and flaccid.

McMurrough and Rose (1967) postulated that these changes in fine structure of the walls from ammonium-ion-limited yeast were due to, the absence of mannan linked glycosidically to other macromolecules, and to a diminished protein content. These workers also suggested that changes in all cell shape during the life cycle could be explained by alterations in cell wall composition at certain loci, resulting in more pliable regions of wall.

Nickerson (1963) suggested that yeast cell wall plasticity is controlled largely by the activity of protein disulphide reductase which calatyses the breakage of disulphide bridges in certain cell-wall proteins. Ammonium-ion limitation has been found to result in lowered protein disulphide-reductase activity in mitochondria from Sacch. cerevisiae (Brown and Hough, 1966), and may account for significant changes in the wall composition of yeast cells grown under conditions of ammonium-ion limitation.

In a study of the amino-acid pool of yeast, it has been shown by Brown and Rose (1969) that, in Candida utilis, the pool size was lower in ammonium-ion-limited cells, than when glucose or phosphate was limiting. Also, the

pool size was found to increase as a result of increased growth rate, lowered oxygen tension within the medium and lowered growth temperature (Brown, 1969).

In an investigation of the water-soluble nucleotides and peptides present in nitrogen-starved yeast, Neuber et al. (1965) found that, while the former increased slightly, the latter increased by more than 50% as a result of nitrogen limitation. This increase in intracellular peptides may help to protect the cell from reactions between carbonyl groups and amino groups of cell protein, a hypothesis put forward by Scott (1960) to explain death in dried micro-organisms. This theory has been supported by Greaves (1960) who found that the addition of amino acids to lyophilisation media as a neutraliser of carbonyl groups resulted in increased cell survival after freeze-drying. A low content of cellular nitrogen has been advocated for the successful lyophilisation of Sacch. sp. (Turui and Ikeda, 1962) and Cryptococcus terricolus (Pedersen, 1965). A significant influence can be exerted by ammonium ions on the steady state of pyrimidine nucleotides in aerobically grown yeast cells, particularly in increasing the amount of reduced intracellular pyrimidine nucleotides (Chance, 1959). A high concentration of ammonium ions in the yeast cell may, therefore, critically deplete the cell of oxidised pyrimidine nucleotides.

Good quality ADY has been shown by Pollock and Holmstrom

(1951) to be associated with high trehalose and glycogen contents. These workers found that good quality ADY could be produced from yeast cells with a total carbohydrate content of 40% and a trehalose content of 16%. The amount of reserve carbohydrate accumulated by yeasts is affected by the concentration of both nitrogen-containing and phosphorous-containing nutrients in the growth medium (Trevelyan and Harrison, 1956a). Trevelyan and Harrison (1956a) also found that anaerobic catabolism of glucose and other sugars was initially accompanied by trehalose breakdown within the cells but that, in nitrogen-free solution this initial breakdown was succeeded by trehalose synthesis. Nitrogen assimilation restricted synthesis of reserve carbohydrates but not the structural cell-wall carbohydrates glucan and mannan. Carbohydrate synthesis was also restricted by high concentrations of phosphate in the solution (Trevelyan and Harrison, 1956b).

More recently, Küenzi and Fiechter (1969), in an investigation into the carbohydrate composition of partially synchronised yeast populations, have shown that the reserve carbohydrates are only accumulated during the single cell phase between two budding periods, and are broken down during bud formation. This evidence is further justification for the use of a 'ripening period' during the growth of yeast cells for ADY production, to ensure the completion of cell division and a high trehalose content within the cells. Glycogen

accumulation under conditions of ammonium-ion limitation has been demonstrated in Escherichia coli (Holme, 1957) and Torula (= Candida) utilis and Aerobacter aerogenes (Herbert, 1961). Synthesis of storage polysaccharides in A. aerogenes has also been shown to be dependant on the growth rate, and the relative balance of potassium and ammonium-ions in the culture medium (Dicks and Tempest, 1967).

METHODS USED FOR PRODUCTION OF ACTIVE DRIED YEAST

The aim of ADY production is to dry the yeast in such a way that all cells remain viable and have maximum fermentative activity after reconstitution. The drying process requires that intracellular water should pass out of the cell through the plasma membrane, and be able to return by the same route without causing gross disruption of the cell structure. Losses in fermentative activity during the drying process have been attributed entirely to changes in the permeability of the plasma membrane (Ebbutt,1961), although, disruption of intracellular organelles and inactivation of enzymes by heat are factors which should not be disregarded. It is with these adverse effects in mind that most commercial drying techniques have been devised, many having been derived empirically as the underlying reasons for their use are still not fully understood (Burrows 1970).

Most commercial ADYs are produced by removing water from a paste of vegetative yeast cells which is divided into small particles that may also contain certain chemical additives. Removal of water is effected by passing warm air around the yeast particles, for periods varying from a few seconds to several hours. One method of yeast preservation has been published in which vegetative yeast cells are induced to sporulate by incubation in a medium containing acetic acid, before being dried (Canadian Patents and Developments Limited, 1969). It is unlikely, however, that the product would be suitable for use in the bakery, although it may be a convenient means by which large commercial yeast inocula can be stored.

When producing ADYs, yeast cells are separated after propagation by filtration methods that are designed to give the highest possible dry matter content. Values between 30 and 38% dry matter are frequently achieved, for example by the use of filter presses. The yeast paste is then converted into small particles, for example 2-25 cubic mm (Hixon, 1922), in an attempt to ensure that, during drying, a uniform rate of water loss occurs from all cells within the particles. This may be achieved by extrusion through a mesh or screen (Walter, 1953) or by comminution in a high-speed macerator (Taylor and Trevelyan, 1974). The latter method had the advantage that the volume of the yeast paste was considerably increased without damage to the yeast cells. Also, the

yeast pellets after drying were found to be of a more regular size and hence more suitable for use in the baking industry. The commercial systems used for the production of ADY have recently been outlined by Burrows (1970), and by Reed and Peppler (1973).

Yeast particles may be dried on static trays (Walter, 1953), on a continuous moving band in a tunnel dryer (Merritt, 1957; Serwinski et al., 1961) or in a rotating drum dryer (Frey, 1957; Thorn and Reed,1959). Active dried yeast produced by the drum-drying method is in the form of hard pellets which are stated to have excellent keeping qualities. Large volumes of air at temperatures ranging from 35° to 90°C are passed over the yeast, but the conditions are so arranged that the temperature of the yeast remains low at all stages, a process that is assisted by evaporative cooling. The drying process normally takes from 6 to 24h, depending on the flow rate and temperature of the air, after which time the water content of the yeast has been lowered to approximately 8% (Burrows, 1970). By close monitoring of the relative humidity of the air which is in equilibrium with the yeast particles, the process can be accurately controlled and the yeast dried to a predetermined water content.

Recently, a variation in these drying techniques referred to as the 'fluidised bed' process, has been adopted for the successful production of ADY (Pressindustria SpA., 1967; Taylor, 1974). In this type of process, particles

of yeast are placed onto a grid or porous platform and warm air is forced up through the bed causing agitation and drying. In a review of the suitability of this process for ADY production, Šestáková (1973a) found that the conventional fluidised bed process did not produce as high a quality product as drum drying. However, the so-called 'vibro-fluidised bed' process, which prevents channelling within the yeast bed, produced high quality ADY without the use of chemical additives.

Another attractive method is spray-drying. This method has been used successfully for dehydrating non-living material such as milk, and has been adapted for the drying of yeast cells. In this system a yeast cream (12-18% dry matter) is sprayed from the top of a tower, and falls through a rising stream of hot air, which removes water from the yeast. The system works most efficiently under short time, high temperature conditions (75-150°C) which give minimum chemical degradation but extensive damage to cellular organelles (Burrows, 1970). The method has the advantage that it is rapid and produces a fine-grained product that is easily reconstituted (Toyo Jozo Co. Ltd., 1965).

Johnston (1963) described a different type of drying process. This involved the suspension of cream yeast in an edible oil such as cotton-seed oil, peanut oil, or soyabean oil. The water was then evaporated by bubbling a gas through the suspension. Best results were obtained using a gas containing some free oxygen. As

in other processes, the rate of drying was controlled by the temperature, flow rate and relative humidity of the gas. The optimal temperature for drying increased from 35-40°C for yeast containing more than 20% moisture, to 60°C for yeast containing from 20-8% moisture. The yeast was then separated by filtration, and residual oils were removed by methyl chloride, hexane, ethanol or acetone to leave smooth-surfaced pellets of ADY.

A method of ADY production has been patented (Brandt and Groth, 1968) that claims to avoid thermal and oxidative denaturation of the dehydrated product. The material for dehydration was spread onto a membrane through which a gas (preferably nitrogen) was forced from below. Microscopic bubbles of gas at ambient temperature were formed and passed through the bed of material causing drying but little oxidative or thermal degradation.

Other methods of ADY production, for example mixing moist yeast with a dehydrating agent such as potassium sulphate, silica-gel, flour or starch, have also been patented (Burrows, 1970). Many compounds have been added to the yeast cream before dehydration to improve the final product. They may be added to; (a) aid the dehydration process; (b) prevent oxidation within the cells (antioxidants), and so extend storage life; or (c) improve water removal and reconstitution (surfactants). However, the precise modes of action of many of the

additives classed as surfactants, especially their relationship with the yeast cell membrane, are not well understood.

Compounds including alkali metal salts (e.g. sodium chloride) and alkali metal salts of a variety of organic acids, have been added to yeast cream before drying in an attempt to ensure rapid, but uniform shrinkage and dehydration within the yeast cell during drying. These additives have been used most successfully during the rapid process of spray drying (Toyo Jozo Co. Ltd., 1965; Toyo Brewing Co. Ltd., 1969; Oriental Yeast Co. Ltd., 1973). It is important to note that the method of Toyo Jozo Co. Ltd. specifically excluded ammonium salts from their list of additives due to their action in decreasing the fermentative activity of the ADY. Other agents that have been added beneficially to yeast during the drying process, include lecithin and carboxymethylcellulose (Toyo Brewing Co. Ltd., 1969), casein, gelatin, albumin and gluten all at a final concentration of 15-25% (Kyowa Fermentation Industry Co. Ltd., 1972), as well as soya protein, mucin, zein, milk protein, alginic acid and polyalcohols (Yakult Honsha Co. Ltd., 1973).

The advantages of adding amino-acids to lyophilization mixtures have already been mentioned. The Kyowa Fermentation Industry Co. Ltd. (1969) have achieved improvements in fermentative activity of ADY in excess of 100% by adding lysine, leucine, isoleucine,

phenylalanine and glutamic acid (0.05-10%) to the yeast cream before drying.

Chen and Cooper (1962) produced a 'Protected ADY' by addition of antioxidants to the yeast cream before drying. Butylated hydroxytoluene or propyl gallate (each at 0.2%, w/w) were found to stabilise ADY in the atmosphere. Chen et al. (1966) in a more detailed investigation compared 'protected ADY' to the normal ADY and confirmed these earlier results and also found that the 'protected ADY' was superior in quality yet had the important economic advantage of not requiring storage under nitrogen. This line of research was also persued by Pomper and Akerman (1969b) who patented the use of a different group of antioxidants including 4-hydroxymethyl-2, 6-di-tert-butyl-phenol. These antioxidants were incorporated into ADYs which as a result then had improved leavening power.

Sucrose diesters, such as sucrose distearate and sucrose dipalmitate, improved the leavening power of the yeast after reconstitution in cold water (Pomper and Akerman, 1968). Pomper and Akerman (1969a) also found that glyceryl diesters with chain lengths greater than 12 carbon atoms imparted superior leavening power to the yeast. Pomper and Akerman (1969a) recommended that the fatty-acyl chains of the glyceryl diester should have between 14 and 18 carbon atoms; it may be significant that most fatty-acyl residues in yeast lipids are of

similar chain length.

Long-chain fatty-acid esters of sorbitol known as Spans provide protection for the yeast cell during drying, while also improving storage, and have been incorporated into ADYs by several manufacturers (Mitchell and Enright, 1959; Chen and Cooper, 1962; Koninklijke Nederlandsche Gist-en Spiritusfabriek N.V., 1973; and the Oriental Yeast Company Ltd.,1973). Mitchell and Enright (1959) incorporated sorbitan-monolaurate,- monopalmitate, - monostearate, - tristearate, - monooleate and - trioleate at an optimal concentration of 2% dry wt. of yeast into their ADY with considerable improvement in retention of fermentative activity during storage. Active dried yeast containing Spans with 18 carbon chains both saturated and unsaturated, showed losses of less than 5% in activity during storage at 46°C for four weeks. Untreated ADY stored under comparable conditions showed considerable loss (approximately 30%) in the same time.

A further advantage of Spans when added to ADY was found by Mitchell and Enright (1959). Their product had a final water content of below 6% (preferably 3%) which was considerably lower than that of active dried yeasts produced by other processes. Such ADY had excellent thermostability when compared with the more common active dried yeasts containing 8% moisture, but required rehydration by water vapour to a water content of 8-10%, before reconstitution in liquid water, if a

satisfactory activity was to be recovered (Mitchell and Enright, 1957). Addition of Spans to ADY permitted direct reconstitution of low moisture ADY in water, with the omission of the rehumidification step (Mitchell and Enright, 1957).

There is evidence to suggest that the most effective Spans used in ADY production are those that contain fatty acyl chains that are, (a) saturated and, (b) of similar chain length to yeast fatty-acyl residues. Chen et al. (1966), in a study of the stability of low moisture (4-8%) ADY, found that sorbitan monopalmitate and sorbitan monostearate (both at 2% level) were the most effective of a variety of sorbitan esters and other adjuncts (containing both saturated and unsaturated fatty-acyl residues) in preventing leakage of cell constituents at both high and low reconstitution temperatures. Furthermore, it was possible to recover at least 95% of the original leavening power of the yeast even after 15 weeks storage under nitrogen at 38°C, which represented a considerable improvement over untreated ADY with a moisture content of 8%.

More recently the Oriental Yeast Company (1973) have found that sorbitan fatty-acyl esters or glycerol fatty-acyl esters added to yeast prior to drying can result in a 25% improvement in recoverable fermentative activity after reconstitution.

Chen et al. (1966) were unable to explain entirely the success in using sorbitan-monostearate and sorbitan-monopalmitate purely by their physical hydrophilic-lipophilic properties. It is possible to speculate that an interaction occurs between the yeast plasma membrane and the Span molecule or any contaminants (e.g. free fatty acids) in the relatively impure commercial ester. As yet, however, there is no firm evidence to support such a theory.

The importance of a high trehalose content in yeast to be dried has been stressed previously. Some workers have found a decided advantage in the addition of fermentable substrates to yeast cream during drying (Johnston, 1963; Toyo Brewing Company Ltd., 1969). These substrates are fermented during the early stages of drying to provide energy which in some way protects the delicate yeast cells from the stress of dehydration. They may also contribute to the store of trehalose within the cells, and certainly release significant quantities of heat to aid the drying process. Johnston (1963) found that when all of the fermentable sugars were provided as supplements the ADY was excellent when freshly prepared. However, the products obtained by drying yeast in the prescence of sucrose, glucose, fructose or mannose were less stable than those obtained with the addition of maltose or raffinose.

The use of certain commercial carbohydrate mixtures

(e.g. acid-hydrolysed corn syrup) was also suggested by Johnston (1963). For the best results it was found that all, or nearly all, of the dextrose should be fermented by the yeast during drying, however, some dextrins and maltose should remain unfermented at levels in excess of 25% by weight of dry yeast. The beneficial effects of carbohydrate fermentation during drying were only observed when the yeast was relatively wet, that is with a water content in excess of 50% (w/w). The effect disappeared once the yeast was dried further.

After production, active dried yeasts are conveniently stored in sealed tins or packets. An increase in loss of fermentative activity can be expected if the containers are not sealed (Šestáková, 1973b). The nature and temperature of the gaseous environment in contact with the ADY can materially alter the storage characteristics of the product. Šestáková (1973b) advocated the use of a vacuum, carbon dioxide, carbon monoxide or nitrogen, and stated that expected losses in ADY activity were approximately 1.5% per month in the presence of nitrogen and 7-8% per month in air. Similarly, Chen et al. (1966) found considerable improvements in ADY storage under nitrogen in sealed containers, alternatively an antioxidant and ester were incorporated into the product.

Felsher (1956) has shown that, when stored in a vacuum, the shelf life of ADY can be extended from 8 days at 50°C, to 21 months at 22°C or even up to 2 years at 4.4°C.

CHANGES IN YEAST CELLS DURING THE DRYING PROCESS AND SUBSEQUENT STORAGE

When yeast cells are subjected to drying, changes occur in the chemical composition, enzymic activity and physical properties of the cells. These changes are affected by the conditions under which the cells have been grown and dried. Such changes are of considerable interest to workers interested in ADY who have been attempting to both improve the quality of the final product and to determine the essential factors causing loss of fermentative activity. The fact that changes occur within the cells as they dry indicates that considerable enzymic activity takes place before sufficient water is removed to render the cells relatively inactive. Some changes also occur during storage of ADY. However it is at present not known whether these are enzyme-mediated or purely chemical in nature.

A high content of carbohydrate is desirable in good quality ADY (Pollock and Holmstrom, 1951). Payen (1949) first showed that enzymic reactions occur during drying by observing that glycogen was converted to trehalose during the drying process. More recently, Bachmann et al. (1973) have carried out a detailed investigation into certain changes that occur in dried yeast cells. They found that, although the total carbohydrate, glycogen and mannan contents fell during drying and during storage, the glucan and trehalose contents increased during drying

and only started to fall after two months storage. The leavening power of the yeast declined during drying by 31%, and 25% in drying processes of 2h and 5h duration respectively, and was further lowered by 63-42% during a six month storage period. Invertase and maltase activities decline during drying and storage whereas protease activity increased during the drying process but fell initially during storage.

In a more recent publication (Bachmann and Kosiek, 1974) these workers state that drying conditions of 4-5h duration at 30-33°C are preferable to more rapid (2h) or lower temperature (20-25°C) conditions for the production of ADY with good keeping qualities. They have also proposed that the drying process is composed of three distinct phases, related to the water content of the yeast cells:

Phase 1: From the start of drying to a water content of 60-55%. Negligible increase in plasma-membrane permeability, proteolytic activity and dehydrogenase activity but a distinct increase in acid phosphatase activity.

Phase 2: Moisture content 55-12%. Further increase in the above-mentioned changes, with an intense peak of dehydrogenase activity at a moisture content of 20-25%.

Phase 3: Moisture content 12-8.5%. Intense increase

in proteolytic activity, permeability of plasma membrane, and a decrease in dehydrogenase activity.

They attributed the three stages of drying to the type of water removed from the cell, that is; stage 1, extracellular; stage 2, intracellular but 'free'; stage 3, intracellular 'bound' water. Their results indicated that the deleterious effects of proteolytic activity on membrane components, and the removal of 'bound' water, essential for maintenance of 'high molecular-fiberous structures' within the cell, were responsible for irreversible changes in the plasma membrane causing greatly increased permeability.

Although of little importance to the baker who uses his yeast once only, the drying process can also produce genetic change within yeast cells. Hieda and Ito (1973) found that the frequency of genetic change in haploid and diploid yeast cells was increased up to 45 fold by freeze-drying and vacuum-drying. Dehydration was known previously to cause a conformational change in DNA structure (Falk et al., 1963). However, this evidence illustrates the far-reaching disruptive effects of dehydration in cell systems.

Although outside the scope of this work there is a large quantity of information concerning the mechanism of death and resistance to drying in bacteria during aerosol

formation. Some of the theories of macromolecular disruption by water removal may, however, be of relevance to ADY (e.g. Webb, 1968).

Gleen et al. (1951) and Notkina (1959) have shown that changes in carbohydrate composition during the drying process are connected with a 30-75% increase in trehalose at the expense of glycogen. Notkina (1959) found that during storage of ADY the change in trehalose content was negligible and could not account for the change in activity of the yeast. Recently, in a study of the changes that occurred in spray dried and layer dried yeast Damberga and Upitis (1973) found that drying caused a loss in protein nitrogen and breakdown of trehalose within the cell, particularly at high temperatures (above 40°C).

Damberga and Laivenieks (1972) have also noted that hot-air drying causes a loss of some 20% of the free lysine contained within cells of Candida tropicalis. By contrast, freeze-drying only caused a 7% loss of lysine. Papova and Damberga (1973) have recently studied the effect of growth conditions and drying on the electrophoretic properties of soluble proteins from Sacch. cerevisiae. They found that cultural conditions had little effect on the quantity or distribution of proteins found in the yeast cells. However, both high and low electrophoretically mobile proteins were sensitive to heat during drying, but it was not possible to correlate the two characteristics.

Harrison and Trevelyan (1963) were not satisfied that the observed decrease in α-glucosidase (maltase) activity, or the presence alone of an autolytic enzyme, could account for the observed dramatic loss in activity associated with the drying of high-nitrogen (8-9%) yeast. In a study of the changes found in yeast lipids during drying, they found no decrease in the contents of fatty-acyl residues, ergosterol or sterol esters. The content of total phospholipids was decreased by 10-20% during drying, the changes being most marked in phosphatidyl-choline and phosphatidylethanolamine. They concluded that loss of activity was due in part to the action of phospholipase 'C' on the phospholipids in the yeast plasma membrane, and that this enzyme was either absent from low-nitrogen yeast or not activated by the drying process. In this context, enzyme inactivation has been shown in rat liver microsomes that have been degraded by phospho-lipases 'C' and 'D' (Lumper et al., 1969). Microsomal membrane degradation, followed by release of [^{14}C] choline or phosphoryl - [^{14}C] choline from microsomes labelled with methyl - [^{14}C] choline, produced extensive inactivation of glucose 6-phosphatase, NADH dehydrogenase, and oxidation of NADPH. Furthermore, these changes could not be reversed by addition of phospholipids.

Removal of water from yeast cells produces a progressive increase in the concentration of high-and low-molecular weight solutes within the cell with a consequent alteration in the extent of molecular interaction.

The stability of DNA is known to be affected by the concentration of cations, and large increases in concentrations of these ions during drying could result in irriversible damage (Leibo and Mazur, 1966). Some salts such as calcium chloride serve as potential destabilisers of native forms of macromolecules as diverse as RNA, collagen and DNA; while others such as ammonium sulphate and potassium dihydrogen orthophosphate, strongly stabilise the native conformation (von Hippel and Wong, 1964).

Any changes in intracellular pH value due to drying may adversly effect membrane stability in ADYs. Indge (1968) has shown that the stability of protoplast membranes of Sacch. cerevisiae is dependant on the concentration of magnesium and hydrogen ions, two factors likely to be affected by the progressive loss of water from the cell.

The physical properties of yeast cells are also subject to change during the drying process. Echigo et al. (1966) showed that the heat resistance of yeast cells depended on their water contents. Heat resistance changed little when the water content was lowered from above 60% to 20%, but increased markedly when the water content was decreased from 20% to 5% (approximately). Koga et al. (1966), made measurements on the physical properties of water in ADY. The equilibrium vapour pressure, heat of vapourisation, dielectric increments and the nuclear

magnetic resonance spectra of partially dried yeast cells were studied in Sacch. cerevisiae with water contents within the range 25-0.08%. It was found that the physical state of the yeast cells could be classified into four regions: The solution region (20-25% water), the gel region (10-20% water), the mobile adsorption region (5-10% water) and the localised water region (0-5% water). Considerable differences in physiological properties (e.g. oxygen uptake) were found between cells in the solution region and those in the gel region. When the yeast passed through the gel region many drastic conformational changes were considered to take place within the cell (e.g. ion pairing or clustering and neutralisation of zwitterions) because, under such conditions, with small dielectric constants, the separately charged conformation of each molecule was energetically less stable than in the wet state. The abrupt changes in the physical properties of the yeast which occurred at 10% and 5% water contents were considered to indicate that the residual water molecules had considerably less rotational freedom than those in bulk water.

RECONSTITUTION OF ACTIVE DRIED YEAST

Before use, ADY must be restored to a hydrated and metabolically active condition, usually carried out by soaking in warm water for a few minutes. Commercial ADY is sensitive to the temperature of the water in

which it is rehydrated. If the yeast is resuspended in water at a temperature of 38-43°C it gives a well raised bread (Cooper and Peppler, 1956), but if the temperature of the water is low (e.g. 5°C) the volume of the loaf is decreased and the bread unacceptable. Peppler and Rudert (1953) first showed the effect of cold water on reconstitution of ADY, the so-called 'cold shock effect'. They showed by plate-counts and staining techniques that about one third of the yeast cells were inactivated by rehydration at 5-10°C. Lowering of leavening power of the yeast was also observed.

Sant and Peterson (1958) showed the interacting effects of the temperature of the rehydrating fluid, and the moisture content of the ADY, on the number of viable cells recovered and their fermentative activity. Suspension of ADY (8% moisture) in water at 4-5°C killed approximately 95% of the cells and lowered their carbon dioxide producing ability by 90% below that of samples rehydrated at 37°C. If, however, the moisture content of the ADY was raised to 25% by vapour rehumidification, suspension in water at 4-5°C had no ill effects. Also, approximately twice as much soluble nitrogenous material was lost from the cells during reconstitution at 4-5°C, than at 37°C. This quantity of nitrogenous material released from cells at 4-5°C represented only 15% of the total cell nitrogen, but represented the majority of the soluble nitrogenous material found within the cell. Similar evidence has

been presented by Ponte et al. (1960) who found that reconstitution of ADY at 5°C and 40°C resulted in leaching from the cell of material that accounted for 29% and 5% respectively of the yeast dry weight. Breads produced from low temperature-reconstituted ADY were poor due to a small loaf volume and poor 'sticky' quality attributed to glutathione leached from the yeast cells.

Mitchell and Enright (1957) have shown that yeast may be dried to a final moisture content of 2-3% without impairing the ability of the yeast to produce carbon dioxide. However, rehydration in a humid environment to a moisture level of 8% was essential before reconstitution in liquid water could take place. Harrison and Trevelyan (1963) have also reported that the optimum reconstitution temperature of an ADY is related to its water content. They cite the case of a yeast in which the optimum temperature was found to range from 21°C at a moisture content of 23% to 42.5°C at 5.8% moisture.

In experiments initiated to explain the influence of rehydration temperature on recovered activity, Herrera et al. (1956) found that approximately 75% of the cell's NAD^+ was extracted by reconstitution at 4.5°C compared with 15% at 43°C. When ADY was rehydrated at 4.5°C the amounts of cell constituents leached from the cells, represented 12% of the total protein, 20% of the phosphorus, 40% of the carbohydrate and 50-60% of the mineral content. These values were two to four times

greater than those obtained for yeast reconstituted at 43°C. Meyerhof and Kaplan (1951) and Rothstein et al. (1959) have also shown that rehydrated ADY becomes permeable to small ions. Rothstein et al. (1959) postulated that the permeability barriers of the cell were destroyed by drying, and that many of the cell components were simply washed out.

Herrera et al. (1956) postulated that formation of a uniform semi-permeable plasma membrane from the structure present in the dry yeast is slow when rehydration is carried out at 4.5°C thus allowing the soluble constituents to escape, whereas rehydration at 43°C leads to a more rapid reconstitution of the plasma membrane with the result that fewer molecules escape. Trevelyan (1966) thought that the plasma membrane could be expected to be entirely disorganised by dehydration. Also he considered that the cell wall became impermeable on drying, and acted as a barrier to leaching thus preventing the loss of soluble cell constituents. He proposed that the extent of leakage on reconstitution was governed by the relative rates of two processes: (a) the re-organisation of the membrane and; (b) the opening of pores in the yeast cell wall. Harrison and Trevelyan (1963) have suggested that the loss in activity of ADY is due to some change in the permeability of the cell membrane caused by chemical changes that occur during drying, and/or a failure of the membrane to reform properly, or quickly enough, when the dried cells are

rehydrated. Losses in activity on reconstitution within the range 7-100% were found in ADY samples with a moisture content of 8.7%. This loss in activity was associated with the loss from within the cells of low molecular weight constituents, including nucleotides. The extent of leakage could be assessed simply and reproducibly by measuring the absorbance of the resuspension supernatant at 260nm.

Ebbutt (1961) noted another consequence of leakage from dried yeast cells during reconstitution. He found that poor quality ADY cells underwent a diminution in mean cell volume of 35% during drying and reconstituion. A close relationship existed between mean cell volume after reconstitution and baking activity. Furthermore, the cell membranes were immediatley permeable to sodium chloride ions when reconstituted, a phenomenon not observed when high-activity dried yeast or fresh yeast cells were mixed with water. He concluded that the permeability of the yeast cell membrane and wall was of primary importance in studies of dried yeast.

Further light on the reconstituion process is given by the surprising results of Echigo et al. (1966), who found that preheating the yeast to 40°C before reconstitution prevented the damaging effects of cold water. Moreover, the beneficial effects of heating persisted even when the yeast was cooled to 20°C before being added to cold water. This protective effect

gradually diminished over a period of about five hours. This observation would indicate the involvement of an effect that is physical and reversible, rather than chemical in nature (Burrows, 1970).

Suomalainen (1968) has attributed the loss in fermenting ability of reconstituted ADY to a shortage of cofactors (e.g. adenosine and nicotinamide nucleotides) caused by leakage from the cells. Vitamins were also leached from cells during reconstitution. Nicotinic acid leakage occurred at both high and low temperatures, whereas thiamin and riboflavin were released more readily at high temperatures. Pretreatment of yeast cells with mercapto-ethanol during sphaeroplast formation, minimised the loss of cofactors. Suomalainen (1968) concluded that leakage of nicotinic acid did not depend on the disintigration of the protoplast.

Ion transport and phosphorylation in rehydrated dried yeast cells have been examined by Riemérsma (1967). Hydrogen ion transport during fermentation of freshly reconstituted ADY was detected, but was not enhanced by the presence of exogenous potassium ions. Potassium ions were not exchanged for hydrogen ions, and inorganic polyphosphate which has been localised at the cell membrane, was no longer synthesied during fermentation.

These data only serve as an indication to the researcher of the complex biochemical and physical changes that

occur within the yeast cell during drying and subsequent reconstitution. It seems likely that many complex changes, rather than one key factor, are responsible for loss of activity. A variation in membrane permeability is certainly one of these changes. One added complication is worthy of note. That is, that the processes of drying and reconstitution are inseparable and neither can be studied in isolation without involving the other. It remains to be seen whether the processes involved in reconstitution are the exact reversal of the changes induced in the cell by the drying process.

YEAST LIPIDS

Although yeast, especially strains of Sacch. cerevisiae, have been studied intensively from a biochemical standpoint, knowledge of their lipids and membranes is not as extensive as that of other cell components, such as nucleic acids, proteins and carbohydrates. It has only been since accurate and sensitive methods for analysis of lipids, particularly thin-layer and gas-liquid chromatography have been available, that reliable data on the lipid composition of yeasts have been reported.

The lipid classes found in yeasts are those typically found in eukaryotic organisms. They include glycerophospholipids, sterols, sterol esters and triacylglycerols (Erwin, 1973). Extensive reviews of yeast lipids and membranes, including the biochemistry of lipid metabolism, have been compiled by Hunter and Rose (1971), and by Rattray et al. (1975).

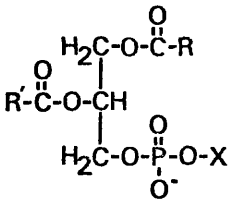
Total Lipid Composition

In general it is possible to recognise two groups of yeasts, based on their total lipid content. The majority of yeasts, including Saccharomyces spp. contain between 7% and 15% dry weight of lipid, while a smaller group, sometimes referred to as 'fat yeasts' contain much more lipid within the range 30%-60% (Hunter and Rose, 1971).

Glycerophospholipids

Glycerophospholipids are substituted fatty-acyl diesters of sn-glycero 3-phosphoric acid. The major phospholipids extracted from yeast cells are phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine and diphosphatidylglycerol (cardiolipin). It is important to note that each of these names refer to a class of phospholipids, individual members of which may vary in the nature of the fatty-acyl residues on the glycerol moiety. Also, numbering of the carbon atoms of glycerol is based on the stereo-chemistry of L-glyceraldehyde (Hirshmann, 1960). Therefore, phospholipids become 1, 2-diacyl-sn-glycerophosphatides, and triacylglycerols are designated triacyl-sn-glycerols (I.U.P.A.C.-I.U.B. Commission, 1967). Figure 1 gives the structures of some glycerophospholipids found in yeasts.

The major phospholipid found in yeasts is usually phosphatidylcholine which represents between 25-50% of the total phospholipids (Jollow et al., 1968; Longley et al., 1968; Getz et al., 1970; Hunter and Rose, 1972). The second major yeast phospholipid is phosphatidylethanolamine, which accounts for 20-32% of the total phospholipid in Sacch. cerevisiae (Longley et al., 1968; Getz et al., 1970; Hunter and Rose, 1972). Both of these phospholipids are also prevelant in Sacch. carlsbergensis (Shafai and Lewin, 1968) and Candida spp. (Kates and Baxter, 1962). Letters (1966) also succeeded in isolating small quantities of



R,R′=Fatty-acyl residues

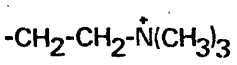
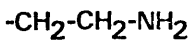
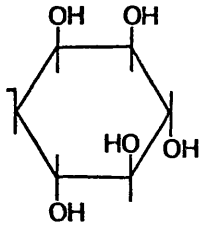
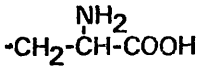
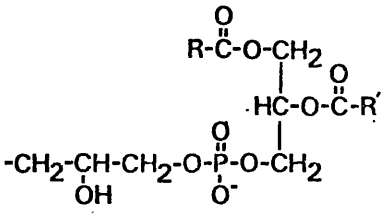
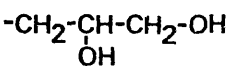
Structure of X	Name of Phospholipid
	Phosphatidylcholine
	Phosphatidylethanolamine
	Phosphatidylmyo-inositol
	Phosphatidylserine
	Diphosphatidylglycerol (Cardiolipin) R,R′=Fatty-acyl residues
	Phosphatidylglycerol

Fig 1 The structures of some glycerophospholipids found in yeasts

N, N-dimethyl- and N-methyl-phosphatidylethanolamine from lipid extracts of Sacch. cerevisiae. These compounds are intermediates in syntheses of phosphatidylcholine from phosphatidylethanolamine (Steiner and Lester, 1970).

Phosphatidyl-myo-inositol was first isolated from yeast and identified by Hanahan and Olley (1958). It is usual to find quite large proportions (about 20%) in Sacch. cerevisiae (Deierkauf and Booij, 1968; Longley et al., 1968; Getz et al., 1970; Hunter and Rose, 1972) and Sacch. carlsbergensis (Shafai and Lewin, 1968). Evidence has been presented for the presence of diphosphoinositides and triphosphoinositides in the lipids of Sacch. cerevisiae by Lester and Steiner (1968) who isolated these two compounds from the deacylated lipids of baker's yeast that were indistinguishable from glycerophosphorylinositol-phosphate and-diphosphate of rat brain. They may be identical with the 4'- and 4', 5'-phosphomono esters of phosphatidylinositol from rat brain. Further evidence for the presence of these compounds in yeast has been presented by Wheeler et al. (1972) who found phosphatidylinositol kinase activity in Sacch. cerevisiae. These di- and tri-esters are however, present in much smaller quantities than phophatidylinositol (Steiner and Lester, 1972).

The proportions of phosphatidylserine in yeast lipids varies. Some yeast such as Lipomyces starkeyi contain relatively large proportions (about 18% of the total;

Hunter and Rose, 1971) whereas Sacch. cerevisiae and Sacch. carlsbergensis contain as little as 4% (Deierkauf and Booij, 1968; Getz et al., 1970; Hunter and Rose, 1972).

Diphosphatidylglycerol(cardiolipin) has been identified in Sacch. cerevisiae by Letters (1966), and usually accounts for some 4% of the phospholipids of this species Deierkauf and Booij, 1968; Getz et al., 1970; Hunter and Rose, 1972). The same lipid has also been found in Candida scottii and C. lipolytica (Kates and Baxter, 1962). Cardiolipin is located mainly in the mitochondria in Sacch. cerevisiae (Jakovcic et al., 1971). Another glycerophospholipid found in similar proportions to cardiolipin is phosphatidylglycerol, which was first isolated from Sacch. cerevisiae by Letters (1966). Phosphatidylglycerol phosphate has been found in lipids extracted from a mutant strain of Sacch. cerevisiae by Deierkauf and Booij (1968) and is beleived to be an intermediate in the biosynthesis of cardiolipin.

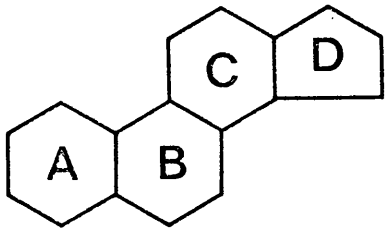
Lysophospholipids are phospholipids which do not possess a fatty-acyl residue on C1 or C2 of sn-glycerol (Tattrie and Cyr, 1963). Many of the lysophospholipids encountered may be artifacts, due to the action of phospholipases during extraction, particularly in anaerobically grown cells (Letters,1968a). Getz et al. (1970) found only 1% of lysophospholipids in Sacch. cerevisiae grown aerobically.

Another phospholipid found in lipid extracts of yeasts which could arise as a result of phospholipase 'D' activity, is phosphatidic acid. This compound has been found in Sacch. cerevisiae (Longley et al., 1968) and Sacch. carlsbergensis (Shefai and Lewin, 1968) but not in C. Lipolytica (Kates and Baxter, 1962).

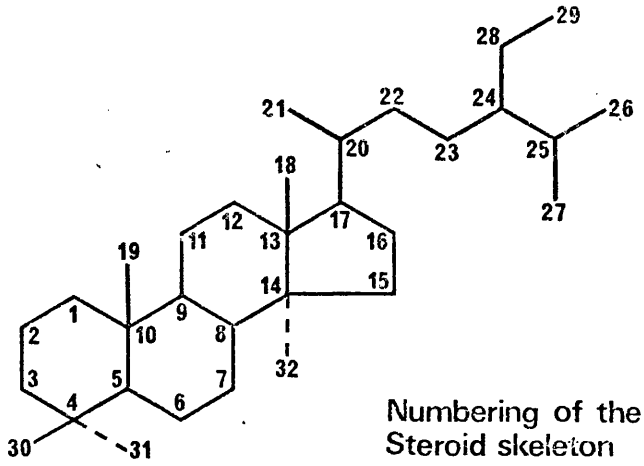
Sterols

Sterols (Figure 2) are based structurally on the cyclopentanoperhydrophenanthrene ring (Fieser and Fieser, 1959; Shoppee, 1964; Klyne, 1965). The total sterol content of yeasts including free and esterified forms usually comprises 0.1-2.0% of the dry weight (Bills et al., 1930; Shaw and Jeffries, 1963), although Dulaney et al. (1954) reported a strain of Sacch. cerevisiae which, under certain cultural conditions could contain 7-10% dry wt. as sterols. Ergosterol (Figure 2) is the principal sterol found in most yeasts which have been studied (Wieland and Benand, 1942; Usden and Burrell, 1952; Dulaney et al., 1954; Hunter and Rose, 1971).

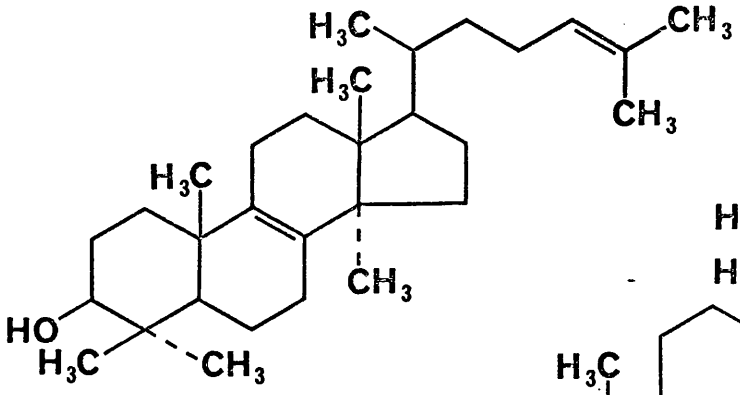
The next most common sterol found in yeast is 24 (28)-dehydroergosterol, first reported by Brevik et al. (1954). This was found to be the major sterol in Sacch. cerevisiae N.C.Y.C. 366 by Longley et al. (1968) although Hunter and Rose (1972) using the same strain found ergosterol and 24 (28)-dehydroergosterol in about equal proportions. Zymosterol has been reported in many yeasts (Dulaney et al., 1954) and it has been proposed as an intermediate



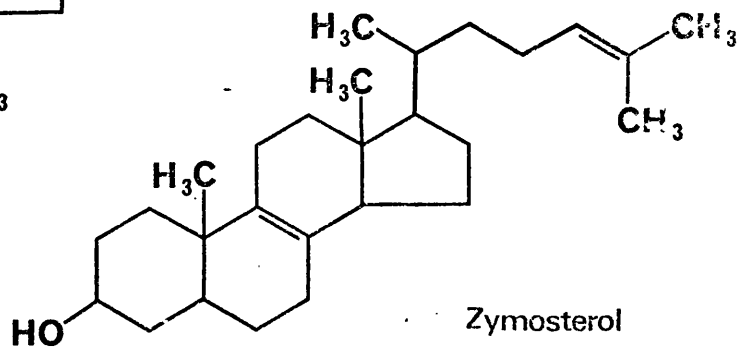
Cyclopentanoperhydro-phenanthrene



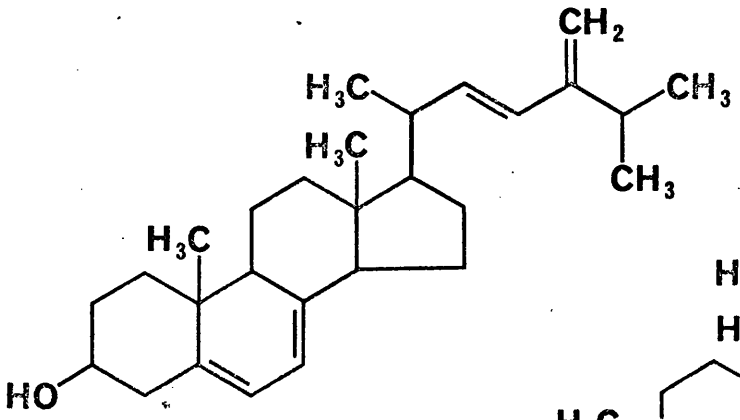
Numbering of the Steroid skeleton



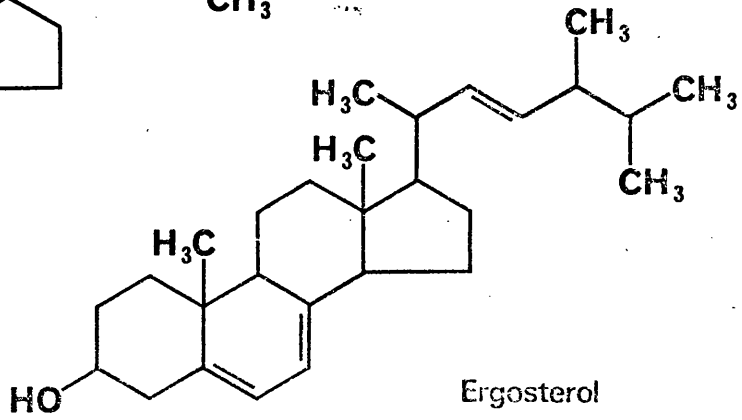
Lanosterol



Zymosterol



24(28)-Dehydro-ergosterol



Ergosterol

Fig 2 Formulae of some important yeast sterols

in ergosterol biosynthesis (Freyberg et al., 1973). Other minor sterols reported in yeast include lanosterol (Weiland et al., 1937), 7, 22 ergostadien - 3 β-ol, episterol, fecosterol (Weiland and Courtelle, 1941), mono- and dimethyl zymosterol (Ponsinet and Ourisson, 1965), and 4α- methyl - 8, 24 (28) ergostadien - 3β -ol (Barton et al., 1968) some of which may be intermediates in ergosterol biosynthesis (Freyberg et al., 1973).

As well as being present as their free alcohols, sterols are also esterified with long chain fatty acids at C-3 (Maguigen and Walker, 1940). Madyastha and Parks (1969) found that C_{16} and C_{18} acids predominated in the sterol esters of Sacch. cerevisiae. An ergosterol-polysaccharide (probably glycogen) complex has also been reported to be present in Sacch. cerevisiae (Adams and Parks, 1968).

Triacylglycerols

These compounds are esters of sn-glycerol and long chain fatty acids. Usually the fatty-acyl residue at C-2 of sn-glycerol is unsaturated as in mamalian lipids (Meyer and Bloch, 1963a). Lipid particles have been isolated from Sacch. cerevisiae in which 90% of the lipid was triacylglycerols together with sterol esters (Clausen et al., 1974). Since they had a high proportion of unsaturated fatty-acyl residues these workers suggested that the lipid particles were reserve materials for membrane biosynthesis.

Diglycerides of 1, 2 - and 1, 3 - diacyl-sn-glycerols and monoglycerides of mono acyl-sn-glycerols have also been found in yeasts (Kates and Baxter, 1962). They are possibly degradation products of triacylglycerols by lipase activity (Nurminen and Suomalainen, 1970) or of phospholipids by phospholipase 'C' activity (Harrison and Trevelyan, 1963)

Fatty Acids and Fatty-Acyl Residues

Free long-chain fatty acids rarely account for more than a few per cent of the total lipid extracted from yeasts. Usually fatty acids occur as residues in phospholipids, triacylglycerols and sterol esters. They may be saturated or unsaturated but are usually straight-chained with an even number of carbon atoms. Chain lengths between 8 and 34 carbon atoms have been reported although chains of 16 or 18 carbon atoms predominate (Hunter and Rose, 1971). Long-chain fatty-acyl residues with 20-34 carbon atoms have been reported in Sacch. cerevisiae, however, they accounted for less than 2% of the total fatty-acyl residues (Welch and Burlingame, 1973).

In a study of the effect of growth temperature on the lipid composition of Sacch. cerevisiae NCYC 366, Hunter and Rose (1972) found that the predominant fatty-acyl residues in whole-cell extracts were $C_{16:1}$, $C_{18:1}$, $C_{16:0}$ and $C_{18:0}$. The unsaturated fatty-acyl residues accounted for 71-77% of the total residues found, depending on the growth conditions employed. Mono enoic residues found

in yeast lipids are usually $\Delta^{9, 10}$ (Hunter and Rose, 1971). Similar results were obtained by Trevelyan (1966) who found a high proportion (80%) of unsaturated fatty-acyl residues in commercial baker's yeast, composed mainly of $C_{16:1}$ and $C_{18:1}$ acids. Residues of $C_{16:0}$ and $C_{18:0}$ accounted almost entirely for the saturated components with only small amounts of odd-carbon numbered acids. Occasionally very small amounts of odd-carbon numbered fatty-acyl residues have been detected (Hunter and Rose, 1972) and one Candida sp. has been reported to contain a small proportion of branched-chained residues (Combs et al., 1968). Branched-chained residues and those which contain cyclopropane rings, which are relatively common in prokaryotes, are absent or rare in yeast.

Polyunsaturated fatty-acyl residues are usually present in only small proportions in yeast (Hunter and Rose, 1971) although $C_{18:2}$ and $C_{18:3}$ fatty-acyl residues represent 35% of the total in kluyveromyces (Sacch.) fragilis (Noble and Duitschaever, 1973).

Other Yeast Lipid Components

Other yeast lipids include hydrocarbons, sphingolipids, polyprenols and glycoplipids. The reader is referred to the account of Hunter and Rose (1971) for a more detailed discussion of these lipid components.

EFFECT OF GROWTH CONDITIONS ON THE COMPOSITION OF YEAST LIPIDS

The lipid composition of yeast is known to vary with changes in environmental conditions. These conditions include age of the culture, growth rate, growth temperature, medium composition and oxygen concentration of the medium.

Age of the Culture and Growth Rate

The lipid content of cells of Candida utilis and Sacch. cerevisiae has been shown to increase with the age of the culture (Dawson and Craig, 1966; Castelli et al., 1969). Of the individual lipid classes, the phospholipids were found to be greatest in content during exponential phase of growth of Sacch. cerevisiae (Castelli et al., 1969; Getz et al., 1970).

Hunter and Rose (1971) have stressed the importance of using chemostat cultures in which the effects of growth rate can be separated from those due to variations in medium composition, growth temperature etc. In such a study these workers found that, with Sacch. cerevisiae grown at 30°C, there was a tendancy towards synthesis of a higher proportion of C_{16} acids as compared with C_{18} acids and also towards production of more unsaturated fatty-acyl residues, the slower the rate at which the cells were grown (Hunter and Rose, 1972). Also, as the yeast was grown more slowly, it synthesised proportionally

more phosphatidylcholine at the expense of phosphatidyl-ethanolamine and phosphatidylserine (Hunter and Rose, 1972). Dawson and Craig (1966) examined the fatty-acyl composition and lipids of Candida utilis and found an increased proportion of $C_{18:2}$ and $C_{18:3}$ residues as exponential growth progressed. They also observed a greater proportion of these residues in Candida utilis grown at a slower growth rate in a chemostat.

Growth Temperature

Lowering the growth temperature caused an increase in total lipid and phospholipid contents of Candida lipolytica (Kates and Baxter, 1962) and Sacch. cerevisiae harvested at the mid-exponential phase of growth (Hunter and Rose, 1972). The latter workers also found that lowering the growth temperature caused a large diminution in the total free and esterified sterol contents. Starr and Parks (1962a) demonstrated that, in Sacch. cerevisiae, the rate of sterol biosynthesis was optimal at 30°C which is the optimal growth temperature. Above 40°C sterol synthesis was inhibited, but addition of exogenous sterol to the culture medium would support growth.

One important effect of lowering the growth temperature is a proportionate increase in the synthesis of lipids containing unsaturated fatty-acyl residues. This effect, which has been observed in many other oganisms is, however, complicated by simultaneous decrease in growth rate of the micro-organism when grown in batch culture.

An increased proportion of unsaturated fatty-acyl residues has been reported in batch-cultures of C. utilis and C. lipolytica (Kates and Baxter, 1962) and in Sacch. cerevisiae by Chang and Matson (1972) who also found an increase in short-chain (Less than 16 carbon atoms) fatty-acyl residues at high temperatures. McMurrough and Rose (1971), however, found only minor changes in the fatty-acyl composition of C. utilis grown at temperatures between 5 and 30°C. Similar results were found by Hunter and Rose (1972) who grew Sacch. cerevisiae at 30°C and 15°C, but at a constant growth rate in a chemostat. Brown and Rose (1969) have also reported an increase in the proportion of $C_{18:3}$ fatty-acyl residues in C. utilis as the growth temperature was decreased, under conditions of both glucose- and ammonium-ion limitation.

Medium Composition

Medium composition is also important in determining the lipid composition of yeast. The total lipid and total fatty-acyl residues increased in C. utilis grown in a chemostat if the glucose content of the medium was increased from 0.1 to 4.5% w/v (Babij et al., 1969). Conversely Brown and Johnson (1970) found that the content of total lipids and unsaturated fatty-acyl residues of Sacch. cerevisiae decline when the glucose concentration was increased. Magnall and Getz (1973) have observed that cardiolipin production is sensitive to the nature of the carbon source in the medium. In Sacch. cerevisiae, transfer

from a glucose-supplemented to a galactose-supplemented medium is associated with an increase in cardiolipin content and respiratory capacity. Jakovcic et al. (1971), however, found similar changes in a respiratory deficient mutant of Sacch. cerevisiae, indicating that loss of respiratory activity does not bear a simple relationship to cardiolipin content.

In a study of the effect of glucose deficiency on the lipid composition of Lipomyces starkeyi, Suzuki and Hasegawa (1974) found that the triacylglycerol content was decreased together with considerable changes in molecular species of phospholipid; notably an increase in the proportion of $C_{18:2}$ fatty-acyl residues.

Vitamin deficiency also affects the lipid composition of yeast. Haskell and Snell (1965) found less total lipid and a lower proportion of unsaturated fatty-acyl residues in Hanseniaspora valliyensis grown in medium deficient in pyridoxine. Similarly, pantothenate - deficient Sacch. cerevisiae has a lower total lipid content than cells grown in the presence of optimal concentrations of this vitamin (Furukawa and Kimura, 1971; Hosono and Aida, 1974). Inositol-deficient Sacch. carlsbergensis synthesises a greater amount of triacyl-glycerols but less phosphatidylinositol than optimally supplemented cells. (Shafai and Lewin, 1968). Biotin deficiency in aerobically-grown Sacch. cerevisiae restricts synthesis of C_{18} fatty-acyl residues, particularly oleoyl residues, while synthesis of C_{16} residues is

proportionately increased (Lynen, 1967).

Phosphate limitation of growth of Sacch. cerevisiae causes an increase in the content of total lipid and fatty-acyl residues, principally in the triacylglycerols, compared with carbon-limited cells (Johnson et al., 1973). Although little change occured in the composition of the fatty-acyl residues, profound changes were observed in the composition of the polar lipids. The levels of the major phospholipids (phosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol) were decreased and replaced by three novel phosphorous-free lipids, possibly containing free amino groups and sugar residues. These workers also reported an increased total lipid content, and fatty-acyl content, together with changes in the fatty-acyl composition, of phosphate-limited cells of C. utilis.

Saccharomyces cerevisiae grown in a rich medium (1.0% yeast extract and 2.0% (w/v) glucose) has been shown to produce more sterol than if grown on less rich medium (0.1% yeast extract and 1.0% (w/v) glucose; Starr and Parks, 1962b).

Oxygen Tension

Yeasts, when grown anaerobically, become auxotrophic for a sterol and an unsaturated fatty acid (Andreasen and Stier, 1954) since biosynthesis of these compounds requires molecular oxygen. The oxygen-requiring reactions in the biosynthetic pathways of these compounds are, respectively, those by which squaline is cyclised (See

Hunter and Rose, 1971), and in which a double bond is introduced into a saturated fatty-acyl residue.

The enzymology of the reactions involved in the desaturation of yeast fatty acids was unravelled by Bloch and his colleagues. Using cell free systems from Sacch. cerevisiae (Bloomfied and Bloch, 1960) and C. utilis (Meyer and Bloch, 1963b) a microsomal enzyme system was isolated which desaturated coenzyme A esters of $C_{16:0}$ and $C_{18:0}$ fatty acids to $C_{16:1}$ and $C_{18:1}$ derivatives, the reactions requiring molecular oxygen (Bloomfield and Bloch, 1960). For introduction of a double bond into the $\Delta^{9,10}$ position in the chain, the enzyme acts on the coenzyme A ester of the saturated fatty acid, and requires NADPH in addition to molecular oxygen, e.g:

$$\underset{\text{(Stearoyl - CoA)}}{CH_3.(CH_2)_{16}.CO.S.CoA} \longrightarrow \underset{\text{(Oleoyl - CoA)}}{CH_3.(CH_2)_5CH{=}CH(CH_2)_9.CO.S.CoA}$$

Serial desaturation gives rise to polyunsaturated acids such as $C_{18:2}$ in C. utilis (Yuan and Bloch, 1961). The enzyme which catalyses these reactions is specific for coenzyme A esters of $C_{18:1}$ acids, and requires molecular oxygen and NADH or NADPH as a cofactor, as well as an unidentified factor, not required for the formation of mono-unsaturated acids (Meyer and Bloch, 1963b). It is worth noting that microsomal fractions of C. lipolytica (Pugh and Kates, 1973) and Torulopsis utilis (Talamo et al., 1973) catalyse desaturation of fatty-acyl residues on phosphatidylcholine and phosphatidylethanolamine, in the presence of molecular oxygen and NADPH or NADH, but

do not require coenzyme A or ATP, as does the synthetase.

Saccharomyces cerevisiae grown anaerobically in medium supplemented with a sterol and an unsaturated fatty acid, has a lower total lipid content than aerobically grown cells (Jollow et al., 1968). Similar observations have been made with a respiratory-deficient mutant of Sacch. cerevisiae (Kovác et al., 1967). The proportions of individual lipids also change in response to anaerobiosis or lowered oxygen tension in the medium. Anaerobically grown Sacch. cerevisiae contains less total phospholipid, sterol and fatty-acyl residues (which contain a higher proportion of short-chain, 10 to 14 carbon atoms, rather than longer-chain unsaturated residues) than aerobically grown cells (Jollow et al., 1968). Candida utilis grown in a chemostat under conditions of glucose limitation, had less saturation in the fatty-acyl residues, together with proportionately more short-chain acids, when the oxygen tension was lowered from 75 to <1mm mercury; the greatest change occurred at tensions of 1mm of mercury and lower (Brown and Rose, 1969). Chang and Matson (1972) found a higher proportion of fatty-acyl residues with 10 to 14 carbon atoms in semi-anaerobically grown Sacch. cerevisiae than in aerobically grown cells. Mono-unsaturated acids ($C_{16:1}$ and $C_{18:1}$) represented about 45% of the total in aerobically-grown cells, but were present in only trace amounts in semi-anaerobically-grown cells.

Of the individual phospholipids, phosphatidylethanolamine

and cardiolpin are found in lower proportion whereas phosphatidylcholine and phosphatidylinositol occur in greater proportions in anaerobically grown Sacch. cerevisiae (Jollow et al., 1968; Getz et al., 1970).

YEAST PLASMA MEMBRANES

Structure and Function

A membrane may be defined as a selectively permeable, pliable barrier surrounding a cell or organelle. Prokaryotic organisms have a relatively simple membrane system consisting of only a plasma membrane, whereas eukaryotes, possess a complex system of intracellular membranes associated with organelles as well as the plasma membrane. The principal functions of the yeast plasma membrane are threefold; firstly, to act as a barrier between the internal environment of a cell and its external environment and so to give it an organised structure; secondly, to regulate transport of compounds into or out of the cell; and thirdly, to be involved in or to provide a site for, certain enzymes such as ATPase associated with active transport, and required for wall synthesis (Matile et al., 1969; Hunter and Rose, 1971).

As long ago as 1899, Overton, from permeability studies on plant tissues, postulated that membranes contained a fatty substance (Overton, 1899). Analysis of membranes have subsequently shown that they are composed mainly of lipid and protein. The major membrane lipids of yeast being phospholipids and sterols. In prokaryotic organisms

sterols are absent (Erwin, 1973).

In 1925 Gorter and Grendel performed the first simple calculation of the amount of lipid in red blood-cell ghosts and postulated enough for the existence of a bilayer. This discovery led to futher research on both the structure and composition of biological membranes. Progress has been greater on membrane composition, while studies of membrane structure have been the subject of considerable debate and criticism (Korn, 1966; 1969).

Two main models of membrane structure have been proposed. Davson and Danielli (1935) suggested that the basic membrane consists of a bilayer some 7.5nm wide, which is seen in thin section in the electron microscope as an electron-transparent lipid layer sandwiched between two electron-dense layers of protein. Robertson (1959) dubbed this as a 'unit membrane'. Although the unit membrane is an attractive model, other theories favour discontinuity within the plane of the membrane. The amphipathic nature of the protein components allows hydrophobic interactions with lipid fatty-acyl chains, while the polar groups could protrude into the aqueous phase on either side (Singer and Nicolson, 1972). This model is known as the 'fluid mosaic model'; (Plate 1).

Most recently, evidence has been presented to indicate that the two halves of the plasma membrane of red blood cells are assymetrical (Freedman, 1975). In a study of

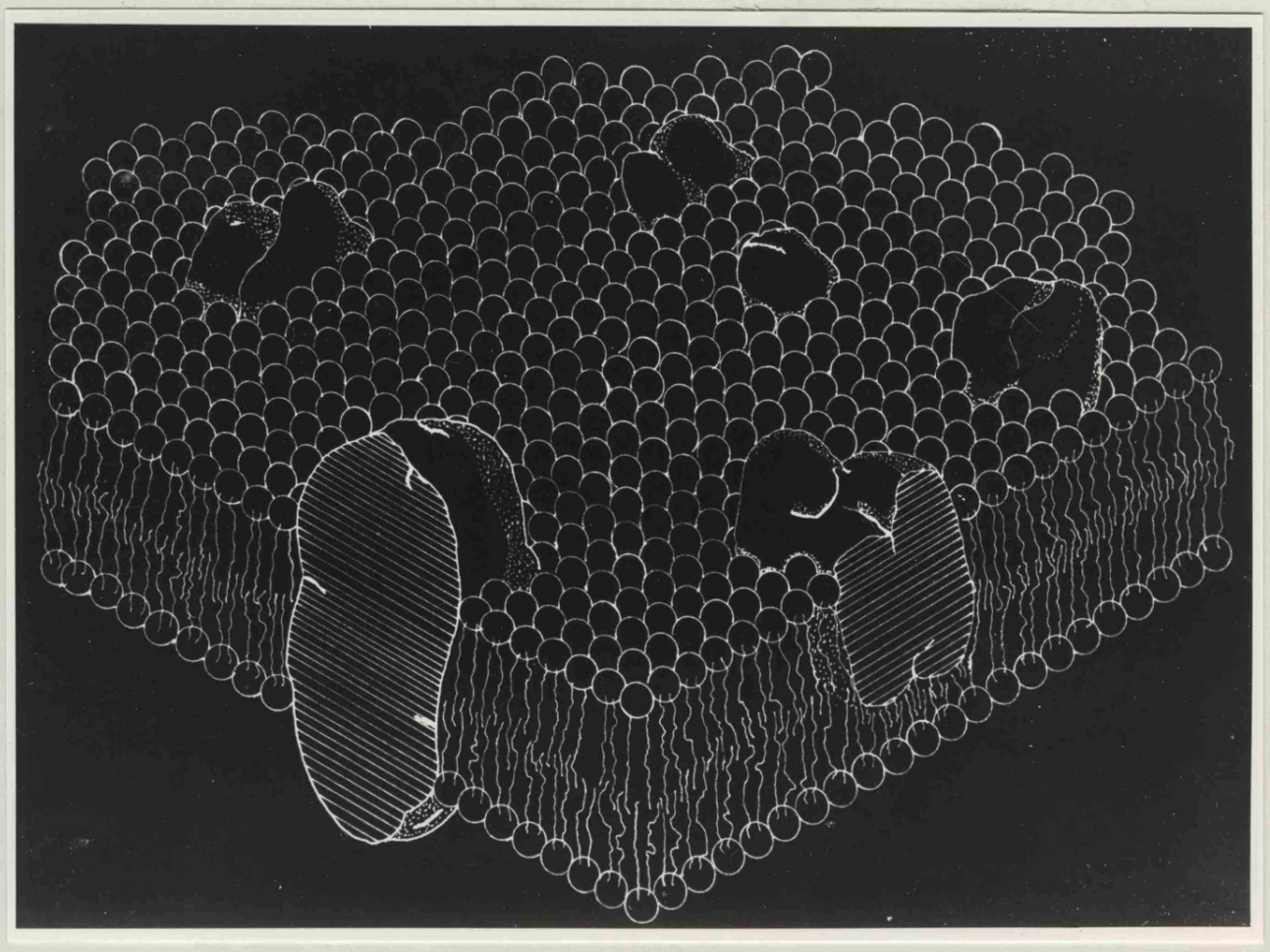


PLATE 1 THE FLUID MOSAIC MODEL

(SINGER AND NICOLSON, 1972)

the effects of certain charged drugs on the structure of red blood cell membranes, Sheetz and Singer (1974) have demonstrated assymetry in the membrane phospholipid content, particularly in the proportions of negatively charged phosphatidylserine within the two layers of the membrane. These workers have presented a general hypothesis with a newly coined name; 'the bilayer couple hypothesis'. Further evidence in support of this hypothesis has been presented by Wisnieski et al. (1974), who demonstrated the occurance of four phase changes in the physical properties of mamalian cell membranes, using electron-spin resonance spectroscopy. These workers explain the data by claiming that two temperatures at which phase changes occur (15° and 31°C) correspond to changes in the outer layer of the membrane bilayer, whereas those at 21° and 37° are attributed to changes in the inner membrane layer.

The reviews by Marchant and Smith (1968), Matile et al. (1969) and Matile (1970) have dealt comprehensively with the structure of the yeast plasma membrane, while a discussion of composition and function in yeast membranes has been presented by Hunter and Rose (1971).

Isolation of Yeast Plasma Membranes

The intracellular membranes in yeast can be demonstrated by electron microscopy (Matile et al., 1969), but no completely satisfactory isolation of plasma membranes has yet been achieved mainly due to difficulties in obtaining

pure preparations.

There are two main methods which can be used to isolate plasma membranes from yeast. Boulton (1965) removed the cell wall of Sacch. cerevisiae with the digestive juice of Helix pomatia (Eddy and Williamson, 1957). The sphaeroplasts so formed were subjected to osmotic lysis, and the plasma membranes and cell debri were obtained by centrifugation at 20,000g for 30 min. Of the membrane fraction 90% of the dry weight was lipid together with protein. Similarly, osmotic lysis has been used on sphaeroplasts of C. utilis by Mendosa and Villaneuva (1967), who confirmed that their membranes consisted mainly of lipid and protein. A more detailed analysis of the plasma membrane of Sacch. cerevisiae isolated by the same method, was carried out by Longley et al. (1968). They found that protein and lipid accounted for 50% and 40% respectively of the dry weight. The remainder comprised nucleic acids and carbohydrate. The lipids were those typical of membranes, namely phospholipids and sterols.

The digestive juice of Helix pomatia is a mixture of some 30 enzymes (Holden and Tracey, 1950), amongst which is known to be a lipase which could conceivably act on the plasma membrane lipid components. To circumvent this problem Nurminen et al. (1970) and Suomalainen and Nurminen (1970) removed the lipase from this juice by gel filtration through Sephadex G.100. Alterthum and Rose

(1973) used an alternative source of glucanase and prepared sphaeroplasts of Sacch. cerevisiae using a partially purified preparation of exo- β -(1-3) glucanase, from Basidiomycete QM 806 (Reese and Mandels, 1959), which is known to be free from lipase activity.

Other workers claim that membranes prepared by osmotic lysis of sphaeroplasts are heterogeneous, and that the analyses carried out do not give representative results. Matile et al. (1967) homogenised cells of Sacch. cerevisiae and subjected the microsomal fraction of the homogenate (separated from the mitochondrial fraction by centrifugation at 150,000g for 30 min) to density-gradient centrifugation using Urografin. The plasma membrane was separated as a distinct band at a density of 1.165 - 1.170 g/cm^3, and subsequent analysis indicated that the major components were lipid and protein together with some carbohydrate. Suomalainen and his co-workers have isolated plasma membrane preparations by digestion, with snail-gut juice, of isolated cell envelopes (i.e. plasma membranes plus cell walls) obtained by breaking baker's yeast in a disintigrator (Nurminen et al., 1970).

The problems associated with obtaining pure preparations of plasma membranes are those of fragmentation (Boulton, 1965) and vesiculation (Dubé et al., 1973). Recently Schibeci et al.(1973) prepared sphaeroplasts of Sacch. cerevisiae with snail-gut juice, and labelled the outer

membrane with different radioactive reagents, selective for various amino-acid residues in the membrane proteins. The sphaeroplasts were washed and the membranes obtained by osmotic lysis and mild mechanical stress. The membrane preparation was layered onto a discontinuous sucrose density-gradient and separated at 189,000 g for 2.5 h. The bulk of the radioactivity was found in bands with a density of 1.18-1.29 g/cm^3. Evidence was presented to suggest a high degree of purity within the preparation, in particular a low level of carbohydrate contamination was recorded.

METHODS FOR EFFECTING CHANGES IN THE LIPID COMPOSITION OF YEAST MEMBRANES

To examine the structural requirements for incorporation of lipids into membranes, any one of the following methods may be used to effect specific alterations in membrane composition; (a) changing the environmental conditions, so that a certain pathway does not function and as a result a specific lipid component is not synthesised. This lipid can then be supplied exogenously in the culture medium; (b) use of mutants deficient in the ability to synthesis certain lipids and which are therefore autotrophic for those lipids; (c) use of drugs as specific inhibitors of a pathway leading to synthesis of lipid. This method, as yet, has found little application.

With reference to the first method, when Sacch. cerevisiae is grown anaerobically it becomes auxotrophic for an unsaturated fatty acid and a sterol (Andreasen and Stier, 1954). A low order of specificity was found for C_{16} and C_{18} acids when provided for anaerobically-grown Sacch. cerevisiae (Light et al., 1962), since acids with a hydroxyl group, a cis double bond, a triple bond, or a cyclopropane ring in the central region of the chain all supported growth. Similarly, Proudlock et al. (1968) reported that the sterol requirement of Sacch. cerevisiae grown under anaerobic conditions, was for a steroid with a planar steroid nucleus, a hydroxyl group at C-3, and a long alkyl side chain at C-17. The nutritional requirements of anaerobically grown Sacch. cerevisiae have been exploited by Alterthum and Rose (1973) and by Hossack (1975) who investigated the effects of altered unsaturated fatty-acid composition and sterol composition on the susceptibility of sphaeroplasts to osmotic lysis.

Changing the fatty-acyl composition of baker's yeast, induced by biotin deficiency, and accumulation within the cells of certain fatty acids exogenously supplied under conditions of biotin limitation, have also been reported by Suomalainen and Keränen (1968).

The use of lipid-requiring mutants provides an elegant means by which whole-cell and plasma-membrane lipid compositions of yeast cells may be altered, but without the need for anaerobiosis. Although sterol-requiring

mutants of Sacch. cerevisiae have been isolated (Karst and Lacroute, 1973; Gollub et al., 1974), most work has been carried out with yeast mutants which are auxotrophic for a fatty acid. The subject has been reviewed by Keith et al. (1973). There are two classes of fatty-acid requiring mutants of Sacch. cerevisiae. Firstly, there are the desaturase mutants, which lack the capacity for desaturating saturated acids ($C_{16:0}$ and $C_{18:0}$) at the $\Delta^{9,10}$ carbon bond. Secondly, there are the chain-lengthening mutants which cannot elongate the fatty-acyl chain.

Desaturase Mutants

Desaturase mutants of Sacch. cerevisiae were first described by Resnick and Mortimer (1966). Since then their fatty-acid specificity and widely variable lipid composition have been of considerable interest to cell physiologists. Desaturase mutants of Sacch. cerevisiae have one important advantage over desaturase mutants of Escherichia coli in that the yeast mutants do not appear to possess any oxidative pathway for metabolising fatty acids as an energy source, as does E. coli. Thus, fatty-acid supplements are incorporated intact into the lipids of the yeast mutant (Keith et al., 1973). It should be noted that data obtained from desaturase mutants of E. coli (Silbert and Vagelos, 1967) and of yeast are not directly comparable as the biosynthetic pathways, and biochemical lesion producing the fatty-acid requirement, are dissimilar (Keith et al., 1973).

The fatty-acid requirements, together with the nature of the genetic lesions of the respiratory-competant mutant yeast strains (KD115, KD20) and respiratory-deficient strain (KD46; Resnick and Mortimer, 1966), have been determined (Keith et al., 1969; Wisnieski et al., 1970; Williams et al., 1973), and may be summarised as follows:

(1) A double bond in the cis configuration at either the Δ^5, Δ^6, Δ^9, or Δ^{11} position, or

(2) A triple bond at the Δ^9 position, or

(3) The Δ^9 trans double bond of $C_{16:1}$ Δ^9 trans, $C_{18:2}$ $\Delta^{9,12}$ trans, trans and, to some extent $C_{18:1}$ Δ^9 trans, 12 hydroxy.

The chain lengths of the fatty acids used in these studies were within the range C_{14} - C_{22} and contained up to five double bonds in the chain.

Genetic analysis indicated that members of the 'KD' series of mutants possessed lesions at two or possibly four distinct genetic loci. The cis configuration was not a strict requirement, although $C_{18:1}$ Δ^9 trans was not adequate to promote growth of mutants. Keith et al. (1973) attributed the general failure of fatty acids containing unsaturations in the trans configuration to promote yeast mutant growth, which they do in E. coli, to the less fluid nature of the hydrocarbon chains at 30°C (optimum growth temperature for yeast), than at 37°C (optimal for E. coli). Further evidence for this explaination has been provided by Esfahani et al. (1969) who found that, when grown suboptimally at 27°C, growth

of the E. coli auxotroph was not supported by $C_{18:1}\ \Delta^{9}$ trans acid.

The effect of fatty-acid concentration on growth rate and cell yield of KD 115 has been reported (Keith et al, 1973). High concentrations (approx. 10^{-3}M) of certain unsaturated fatty acids were inhibitory although growth supporting or inactive at lower concentrations. Effects of this type were attributed by Keith et al. (1973) to variations in chain length or degree of unsaturation, however, these workers do not seem to have acknowledged the possibility of oxidation of unsaturated fatty acids, resulting in formation of toxic by-products,being a significant factor; (see discussion).

In general, the ability of an unsaturated fatty acid to promote growth of strain KD46 (measured as the number of yeast cells produced per femtomole of fatty-acid supplement)has been shown to increase with the number of double bonds. Values for the acids used varied from 1.7 to 11 cell/f mole, with values for $C_{18:1}\ \Delta^{9}$cis, $C_{16:1}\ \Delta^{9}$cis and $C_{22:6}\ \Delta^{4,7,10,13,16,19}$ at 2.7, 4.3 and 11.0 cells/f mole respectively (Barber and Lands,1973).

The percentage incorporation of fatty-acid supplements in the lipids of yeast cells can be as high as 90% (Keith et al., 1973). Levels of fatty-acid incorporation within the range 40 - 75% are frequently acheived but depend on the yeast strain and fatty acid used, together

with the physiological state of the cells when harvested (James et al., 1972; Wisnieski and Kiyomoto, 1972).

Chain-Lengthening Mutants

Mutants of Sacch. cerevisiae requiring long-chain saturated fatty acids have been isolated by Schweizer and Bolling (1970), and by Henry and Fogel (1971). These strains exhibit optimal growth only if the medium is supplemented with long-chain fatty acids with between 12 and 18 carbon atoms. Schweizer and Bolling (1970) purified the fatty-acid synthetase system of their mutant strain and found that, of the seven reaction steps involved, the ability to condense acetate and malonate to acetoacetate was completely lacking. The other six biochemical steps were identical in specific activity with those of the wild-type strain. They concluded that the mutant possessed a missense mutation in the structural gene of either the condensing enzyme, or the 'acyl carrier protein'. More recently, Schweizer et al. (1973) made use of similar mutants to determine some genetic, biochemical and protein structural characteristics of yeast fatty-acid synthetase system.

Henry (1973) demonstrated that logerithmic death of fatty-acid synthetase mutants occurs in unsupplemented medium. This phenomenon did not occur when desaturase mutants of Sacch. cerevisiae were starved of unsaturated fatty-acid. This difference, she concluded, was because the desaturase mutant, though starved of unsaturated acid, was still able to maintain cellular processes

with a decreased level of unsaturation in the cellular lipids. The fatty-acid synthetase mutant, however, was completely deprived of fatty acids under starvation conditions and could not maintain its membrane systems. Treatment of such cells with detergents resulted in accelerated viability loss.

More recently, the complex nature of the genetic lesions in fatty-acid-requiring mutants has been illustrated by Meyer and Schweizer (1974). They described a yeast mutant that required a saturated C_{12}-C_{14} acid for growth at 37°C, yet at 22°C the same fatty acids were not required, and supplementation with $C_{16:0}$, $C_{16:1}$ or $C_{18:1}$ fatty acid caused inhibition of growth. These workers postulated that the exogenously supplied fatty acids acted as allosteric effectors (or in certain cases inhibitors) for a mutationally altered cellular protein in the fatty-acid synthetase complex.

The work reported in this thesis concerns the effects of altered fatty-acyl composition of cell lipids and plasma membranes, on the recovery of fermentative activity after drying in Saccharomyces cerevisiae.

METHODS AND MATERIALS

ORGANISMS

The strains of Saccharomyces cerevisiae used in this study were derived from a sample of commercial active dried yeast (ADY) supplied by the Distillers Company Limited (Glenochil Technical Centre, Menstrie, Clackmannanshire, Scotland). The strains were designated as follows:

Parent strain; A single-cell isolate of the commercial ADY strain obtained from a plate containing glucose-salts-vitamins agar (Diamond and Rose, 1970) on which a suspension of ADY cells had been streaked. The ADY was reconstituted in water at 38°C for 15 min.

HP 92 strain; A mating strain derived from one ascospore, obtained by micro-dissection of an ascus produced by the Parent strain.

RP 108 strain; A mating strain that requires an unsaturated fatty acid for optimal growth. It was derived from strain HP 92 following treatment with N-Methyl-N-Nitro-N-Nitroso-guanidine (MNNG). Details of the mutagenisis procedure are recorded later in this section.

Original cell isolates used in this study were maintained at -196°C in vials under liquid nitrogen. Approximately 1×10^9 washed stationary-phase cells were resuspended in 1ml 15% (w/v) glycerol solution and sealed in 2ml glass

ampoules. Ampoules were cooled at approximately 1°C/min by lowering into the gaseous phase of a liquid-nitrogen container (British Oxygen Ltd., Dear Park Rd., London S.W.19) until a temperature of -70°C was reached. Cooling was controlled manually by reference to a suitable temperature recorder. Ampoules were then plunged into the liquid phase of the container to complete the cooling process. An approximate one hundred-fold decrease in numbers of viable cells was recorded following the immediate revival of cells at 30°C. The extent of this decrease in viability was not significantly changed over a period of several months.

At intervals during this study, cells were revived at 30°C from the ampoules stored under liquid nitrogen and maintained at 4°C on slopes of yeast extract (1%, w/v), mycological peptone (2%, w/v), dextrose (2%, w/v), agar (2% w/v), (YEPD) supplemented with Tween 80 2% (v/v) if appropriate (Resnick and Mortimer, 1966).

METHODS USED TO PRODUCE MATING STRAINS

Induction of Sporulation

Cells were grown in a presporulation medium containing 1.3% (w/v) nutrient broth, 5% (w/v) glucose and 1% (w/v) yeast extract; pH 6.3 (Fowell, 1969). Portions (100ml) of medium were dispensed into 250ml conical flasks, plugged with cotton-wool and sterilised at 115°C for 15 min. Batches of medium were inoculated with a loopfull of Parent cells from a slope culture and incubated at

30°C in a Gyrotary shaking incubator (Gallenkamp and Co., Technico House, Christopher Street, London E.C.2) with a displacement of approximately 3cm and a shaking rate of 200 rev./min. After 40h the culture was in the stationary phase of growth (approximately 6.0mg dry wt. equiv./ml). The cells were harvested by centrifugation for 5 min at 12,000g at 5°C, washed twice with water and resuspended in a small volume of water.

Sporulation of cells was induced by incubating vegetative cells in a sporulation medium containing 0.5% (w/v) sodium acetate and 1.0% (w/v) potassium chloride;pH 7.0 (Fowell, 1969). Batches of sporulation medium (100ml in 250ml conical flasks) were inoculated with vegetative cells to a density of 0.3 - 0.4mg dry wt. equiv./ml and incubated at 25°C in a Gyrotary shaking incubator as previously described. Ascospores were visible microscopically after approximately 30h incubation, and spore formation was complete after about 48h. However, the suspensions were incubated for up to 120h to allow the spores to ripen before harvesting by centrifugation at 12,000g for 5 min at 5°C. The mature asci were washed twice with water using identical centrifugation conditions.

Enzymic Dissolution of Ascan Walls prior to Ascospore Separation

Mature asci were resuspended to 10mg dry wt. equiv./ml in water and 4ml of this suspension together with 1ml of Basidiomycete glucanase (See Preparation of

Sphaeroplasts) containing 200 units (equivalent to 5.0 units/mg dry wt. equiv. cells) were added to a 25ml conical flask and the mixture incubated at 30°C in a water bath with gentle reciprocal shaking. Ascan wall digestion was observed microscopically and, when complete after approximately 30 - 50 min, the ascospores were harvested by centrifugation at 1,500g for 10 min at 20°C and washed once with sterile distilled water.

Microdissection of Asci

The apparatus required, and the techniques used for the microdissection of asci, have been described in detail by Fowell (1969). A brief summary of the method is given below.

A small volume of glucanase-treated ascan suspension was streaked onto the edge of a previously prepared MYGP agar film approximately 0.2mm thick supported on a sterile coverslip (22 x 33mm; 0.2mm thick). The composition of the MYGP agar was as follows; malt extract 0.3% (w/v), yeast extract 0.3% (w/v), mycological peptone 0.5% (w/v), glucose 1.0% (w/v), agar 2.0% (w/v); pH 5.0. The coverslip was inverted and attached by its edges with clear nail varnish to the top surface of a 'U' shaped moist chamber containing a pad of damp filter paper. Thus the agar film and asci were within the moist chamber suspended by the coverslip. The moist chamber was placed on a fixed-stage moving-objective microscope and the smear of asci brought into view.

Rupture of selected asci was carried out using a microneedle (end diameter, 30-100 μm), attached to a De Fonbrune pneumatic micromanipulator. The microneedle was introduced into the moist chamber and adjusted until the tip was just touching the surface of the agar film near the smear of asci. Asci containing four mature ascospores were pulled away from the ascan smear using the microneedle, to regions of the agar film previously allocated and marked by a set of four ink spots on the top surface of the coverslip. Each ascus was gently rolled on the agar surface until it ruptured. The ascospores were separated and each placed close to one of the four ink spots.

Isolation of Strains Derived from Individual Ascospores

After micromanipulation the coverslip, together with the agar film, was carefully removed from the moist chamber and secured, agar film upwards, to the inside of the lid of a sterile petri dish, using varnish. Moistened filter paper was placed in the base of the dish to prevent desiccation of the agar film and the whole dish incubated at 25°C for 18-24h during which time germination and micro-colony formation took place. Using the ink spots as a guide, the agar film was dissected using a sterile scapel, and individual ascospores or microcolonies were removed using a microspatula and incubated in 2.5ml MYGP broth in Bijou bottles at 30°C for 48h. In bottles in which growth had occured, the clone was isolated onto MYGP-agar slopes, incubated at

30°C for 48h and then stored at 4°C.

Mutagenesis

Volumes (100 ml) of YEPD broth in 250ml conical flasks were inoculated with cells of a selected mating strain (see Results) from YEPD agar slope culture. The cultures were incubated at 25°C for 15 - 18h in a Gyrotory shaking incubator as previously described. The cells were harvested by centrifugation for 5 min at 12,000g at 20°C, washed twice with 0.2M sodium acetate buffer (pH 5.0), and resuspended in that buffer at a density of 3×10^8 cells/ml (Megnet, 1965). A portion (1.8ml) of this cell suspension together with 1.2ml of MNNG solution (3mg N-Methyl-N'-Nitro-N-Nitroso-guanidine per ml 0.2M sodium acetate buffer; pH 5.0) were incubated in a 25ml universal bottle for 30 min at 30°C with gentle agitation. The reaction mixture was then diluted into 150ml 67mM phosphate buffer (pH 4.5) and the yeast cells recovered by centrifugation at 6,000g for 10 min. The yeast sediment was washed once with a similar volume of 67 mM phosphate buffer.

Isolation of Auxotrophic Mutants

Yeast cells that had been treated with MNNG were resuspended in 100ml of YEPD + 0.2% (v/v) Tween 80 in 250ml conical flasks and incubated for 6h at 30°C in a Gyrotory shaking incubator. The cells were centrifuged and washed twice with 100ml 67mM phosphate buffer, resuspended in a small volume of buffer and stored at

4°C for 48h. Meanwhile, the number of viable cells in the culture containing the MNNG-treated cells was determined by serial dilution of a sample in 67mM phosphate buffer, and plating out on YEPD agar including 0.2% Tween 80 plates, followed by incubation at 30°C for 48h.

The concentration of viable MNNG-treated cells in 67mM phosphate buffer was adjusted to give 100-150 viable cells/0.1ml and a large number of Petri dishes containing YEPD agar supplemented with 5 μg/ml Tween 80 were spread each with 0.1ml of yeast suspension. The Petri dishes were incubated for five days at 30°C. Cells from pin-head colonies were inoculated, using wooden tooth-picks, onto Petri dishes containing; (a) YEPD agar without Tween 80 and (b) YEPD agar supplemented with 0.4% (w/v) Tween 80. All plates were incubated at 30°C for 48h. Isolates that produced colonies on Tween 80-supplemented medium but not on unsupplemented medium were grown on slopes of YEPD agar containing 2% (v/v) Tween 80 at 30°C for 48h and maintained at 4°C for further study.

EXPERIMENTAL CULTURES

During this study two different culture systems were employed for production of yeast cells; (a) batch cultures (100ml in 250ml conical flasks) were used in experiments to determine the fatty-acid requirement of strain RP 108, and for production of suitable inocula

for the large-scale growth system; and (b) a second system was developed capable of producing cultures with a high density of cells (up to 16.0g dry wt. equiv./litre), suitable for conversion to active dried yeast. By regulating the supply of nitrogen-containing nutrients and other nutrients in the form of concentrated medium solutions in the second system, it was possible to control the total nitrogen content of the cells and also restrict their growth rate; these two properties being essential for the successful production of active dried yeast (Burrows, 1970).

Small-Scale Batch Cultures

Portions (100ml) of yeast extract-glucose-salts-vitamins medium, referred to as medium S, were dispensed into 250ml conical flasks which were then plugged with cotton wool and autoclaved at 115°C for 10 min. The composition of medium S (pH 5.0) per litre of distilled water was:

Yeast extract	10.0g
Glucose	51.3g
$(NH_4)_2SO_4$	6.0g
$NH_4Fe(SO_4)_2$	30.0mg
K_2SO_4	2.0g
$ZnSO_4$	10.0mg
d-Biotin	100.0µg
D-Pantothenic acid Ca salt	1,600.0µg
i-Inositol	75.0mg
Succinic acid	6.296g
NaOH	2.133g

pH value adjusted to 5.0 with NaOH.

With the exception of Tween 80, all supplements were added to the basal medium after sterilisation. The detergents Tergitol NPX and Triton X-100 were added as small volumes (0.1-1.0ml), undiluted or in suitable aqueous dilutions, having been previously autoclaved, at 115°C for 10 min.

Initially the addition of small quantities of fatty acids to the basal medium after autoclaving presented a problem, since all of the long-chain fatty acids used in this study are insoluble in water. At an early stage, it was decided to avoid possible deleterious effects of heating and oxidation of fatty acids, particularly the unsaturated ones, during autoclaving. Accordingly, ethanol was chosen as the most suitable solvent in which to dissolve the fatty acids. However, it proved impossible to sterilise ethanolic solutions by membrane filtration due to solubilisation of the plastic membrane. Stock solutions up to a concentration of 500mg of various fatty acids/ml were therefore prepared as follows: Fatty acid was transferred to a sterile volumetric flask, dissolved in boiled ethanol (95%,v/v) and made up to volume with boiled ethanol. Ethanolic stock solutions were stored in the dark at -20°C and the head space in flasks containing unsaturated fatty acid was quickly flushed with nitrogen gas after opening. Periodic sterility checks were made, employing standard plating techniques using malt-extract agar and nutrient-agar media. These tests indicated that the ethanolic

stock solutions remained sterile under these storage conditions. In addition, ethanolic solutions of the anti-oxidants α-tochopherol and butylated hydroxy-toluene (BHT) were prepared and added in similar small volumes (0.1-1.0ml) to the basal medium as required.

Cultures set up to provide a suitable quantity of yeast cells for inoculating large-scale one-litre cultures were inoculated directly from a suitable slope culture and incubated at 30°C in a Gyrotory shaking incubator at 300 rev./min. The cultures reached stationary phase at a cell density of 5.0 - 8.1mg dry wt./ml after 24 - 36h depending on the strain used, and were harvested by centrifugation in an M.S.E. High Speed '18' refrigerated centrifuge at 20°C for 5 min at 12,000g. Cells were washed once with water at 20°C.

Cultures used for studying the fatty-acid requirement of strain RP 108 were inoculated with between 0.3 and 0.4mg dry wt. equiv. yeast/100ml, unless otherwise stated. This inoculum was prepared by incubating a suitable quantity of cells from a slope culture (supplemented with unsaturated fatty acid) in un-supplemented S medium at 30°C for 12 - 16h, washed twice in water, and harvested by centrifugation as already described. In this way, the cells used for the inoculation of experimental cultures were starved of unsaturated fatty acid, and the possibility of any subsequent carry-over minimised. Experimental cultures

were inoculated at 30°C as already described for periods up to 100h.

Large-Scale Batch Cultures

The culture vessel (Figure 3) consisted of a 2 litre round flat-bottomed flask containing a 4.0 x 0.9cm Teflon magnetic follower and plugged with a rubber bung holding five glass entry ports constructed from small-bore glass tubing (4mm internal diameter). Three ports, two for media input and one for air outlet via a cotton-wool plugged filter, protruded just into the neck of the flask. Of the two remaining ports, one carrying the sterile air inlet was positioned deep in the vortex formed by the magnetic follower, while the second was positioned in the culture medium enabling small volumes (up to 10ml) to be sucked over into a sterile universal bottle for routine sampling.

Cultures were incubated at 30°C by placing the flask in a small perspex water bath, with water circulation at 30°C, positioned over a magnetic stirrer (Gallenkamp) at 1,200 rev./min. (Patching and Rose, 1969). The air supply for cultures was taken from a 2.1 x 10^5 Pa compressed-air line, metered through a flow meter (0.2 - 2.0 l/min; G.A. Platon Ltd., Wella Road, Basingstoke, Hants.) set at 1 litre/min, and filtered through cotton-wool and a 'Microflow' filter capsule (Microflow Ltd., Mackley Filter Divison, Minley Rd., Fleet, Hants.).

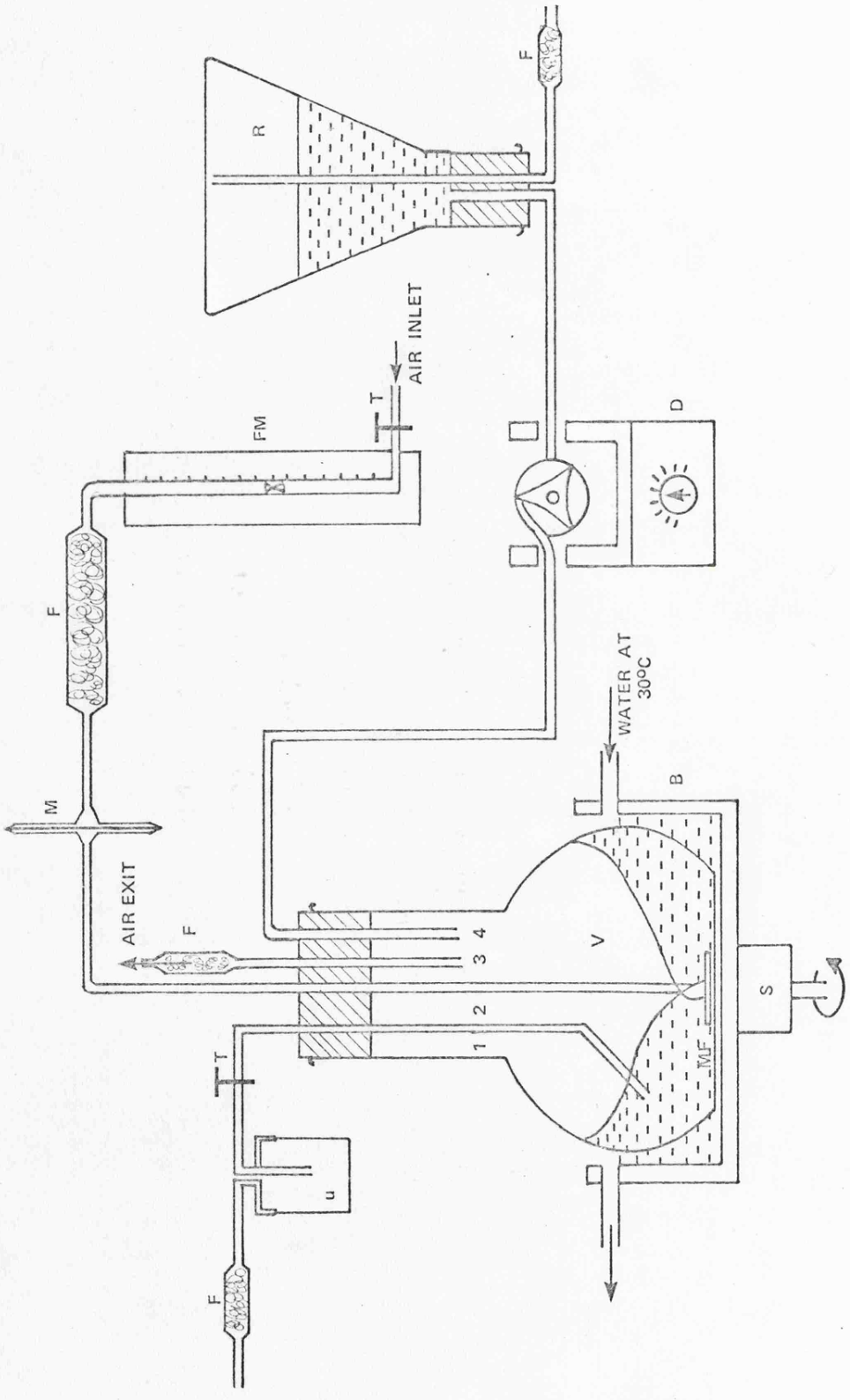


Figure 3 Diagram of Large-Scale One-Litre Culture Apparatus (Not to Scale)

Key to figure 3

B	Water bath
D	Watson Marlow Delta pump with variable speed facility
F	Cotton-wool-plugged filter
FM	Gas flow meter
M	Microflow filter
MF	Magnetic follower
R	Reservoir for medium feed (two)
S	Magnetic stirrer
T	Tap
U	Universal bottle for sampling
V	Growth vessel
	Ports (1) Sampling
	(2) Air inlet
	(3) Air outlet
	(4) Feed medium inlet (two)

Media-feed reservoirs (inverted 500ml conical flasks plugged with rubber bungs; Figure 3) were connected to the culture vessel via a peristaltic pump (Watson Marlow Delta type MHRE; Watson Marlow Ltd., Falmouth, Cornwall). Each medium reservoir had two ports constructed from glass tubing. One, the medium outlet, was set flush with the inside surface of the bung to ensure delivery of the entire volume of medium within the flask, while the second port extended almost to the bottom of the flask and allowed equilibration of the head space with atmospheric pressure, via a cotton-wool-plugged filter.

Use of different size silicon-rubber pump tubing, within the range 1.0 - 3.0mm internal diameter, together with the variable speed facility of the MHRE Delta pump, enabled the rate of each medium supply to be controlled within the range 0.05 - 0.4ml/min. Each individual section of silicon-rubber pump tubing was calibrated after twice being autoclaved at 121°C for 15 min during which time significant stretching occurred. Suitable pump-flow rates were then obtained for each section of tubing from a calibration curve which related flow rate to the pump setting. All connections made between the various components of the complete culture apparatus were with silicon-rubber tubing. The complete culture apparatus was autoclaved at 121°C for 15 min before use.

The compositions (per litre of distilled water) of each of the three media used for the production of yeast in the 1 litre large-scale culture system were as follows:

Start medium (St)

Medium G (without sodium bicarbonate)	10ml
Mono ammonium phosphate	0.8g
$MgSO_4.7H_2O$	0.1g

Sterilised by autoclaving 500ml quantities at 121°C for 15 min.

Nitrogen feed medium (N)

For growth of the Parent strain	13.5g	ammonium sulphate
For growth of mating strains	11.5g	ammonium sulphate

Sterilised by autoclaving 250ml quantities at 121°C for 15 min.

Nutrient feed medium (G)

Glucose	205.2g
$MgSO_4.7H_2O$	1.2g
$CaCl_2$(anhydrous)	0.12g
Ferric ammonium sulphate	0.16g
$ZnSO_4.7H_2O$	0.6g
$CuSO_4.5H_2O$	0.2mg
Thiamin HCl	6.5mg
Riboflavin	9.0mg
d-Biotin	0.4mg
Nicotinic acid	80.0mg
p-Amino benzoic acid	20.0mg
Pyridoxine HCl	7.0mg
D-Pantothenic acid Ca salt	32.0mg
i-Inositol	0.5g
K_2SO_4	5.42g
Succinic acid	12.60g
NaOH	26.67g

pH value adjusted to 5.0

Medium G was dispensed in 125ml portions and stored at -20°C. Before use each portion was added to an equal volume of sodium bicarbonate solution containing (per litre of distilled water);

For the growth of the Parent strain	45.00g
For the growth of mating strains	38.34g

The medium was then sterilised by membrane filtration (0.45 μm diameter pores; Oxoid) immediately before use.

Yeast was grown in the one-litre scale culture system using the following procedure: Portions (500ml) of St medium containing fatty acid supplement, if appropriate, were allowed to equilibrate in the 2 litre culture vessel with the water bath at 30°C. The stirring rate was 1,200 rev./min and the air flow rate 1 litre/min. Cultures were inoculated with cells grown as previously described, to a cell density of 1.0 $\pm$ 0.1 mg dry wt. equiv./ml. Portions (250ml) of the media N and G containing bicarbonate were then passed into the culture vessel at a constant rate over a period of 24 - 36h. The precise flow-rate of each medium feed and the duration of incubation depended on the yeast strain used, (See Results section). Yeast cultures were incubated at 30°C with aeration for a further 60min after cessation of media additions.

Cells were harvested at 12,000g for 10min at 20°C, and were washed three times (unsupplemented cultures) or five times (fatty-acid supplemented cultures) with distilled water (250ml).

Growth Estimation

Growth in both small and large scale cultures was monitored turbidimetrically using either a Hilger 'Spekker' absorptiometer or a Pye-Unicam SP 600

spectrophotometer at 520nm. With each instrument suitable calibration curves relating absorption and dry weight were prepared (See Yeast Dry Matter). Separate curves were prepared for Parent and mating strains of yeast. However, the curve prepared for the Mating Parent strain (HP 92) was virtually identical with that prepared for the mutant strain (RP 108) grown in S medium supplemented with $C_{18:1}\ \Delta^{9}$ cis fatty acid (10^{-3}M), and so this curve was used throughout for both mating strains at all concentrations of fatty-acid supplementation.

Sterility and Reversion Checks

All experimental cultures were examined microscopically for the presence of contaminants. Before harvesting, volumes (0.1ml) of each experimental culture were spread on nutrient agar plates (Oxoid) which were then incubated at 30°C for two days and examined for growth of bacteria. Portions (0.1ml) of culture fluid, together with suitable dilutions in 67mM phosphate buffer (pH 4.5), containing fatty-acid requiring mutant cells, were spread on petri dishes containing YEPD plus agar (2%, w/v) and YEPD agar plus Tween 80 (2%, v/v) incubated for two days at 30°C and examined for the presence of revertant colonies.

Screening for Respiratory Deficient Strains

The Parent strain, mating strains and the auxotrophic mutant strain RP 108 were tested for respiratory deficiency using the method of Nagai (1963). Cells

were streaked onto YEPD agar medium supplemented with Eosin (10mg/litre) and Trypan Blue (15mg/litre). Plates were incubated at 30°C for upto five days. Respiratory-competant strains produced grey-pink colonies, whereas respiratory-deficient strains produced small brilliant purple colonies.

PRODUCTION OF ACTIVE DRIED YEAST

The method used for production of active dried yeast (ADY) was designed to simulate on a laboratory scale a method used commercially. The method chosen was that frequently known as 'tray drying', in which batches of 'cake' or 'wet' yeast (30 - 35% approx. dry wt. equiv.) are held stationary in a stream of relatively warm dry gas (usually air) for a period of hours, during which time both extracellular and intracellular water is removed from the yeast, until a final water content of approximately 90% is reached.

Construction of the Drying Cabinet

The cabinet was constructed of 2.0cm thick blockboard lined internally with expanded polystyrene sheet (approx. 0.6cm thick) and aluminium foil, to give a gas-tight cabinet with external dimensions of 0.91 x 0.65 x 0.36 metres, and a volume of 162 litres. Perspex sheet (0.6cm approx.) and aluminium sheet (0.2cm approx.) were used to construct the door (double-glazed), and the internal shelving and partitions. (See figure 4

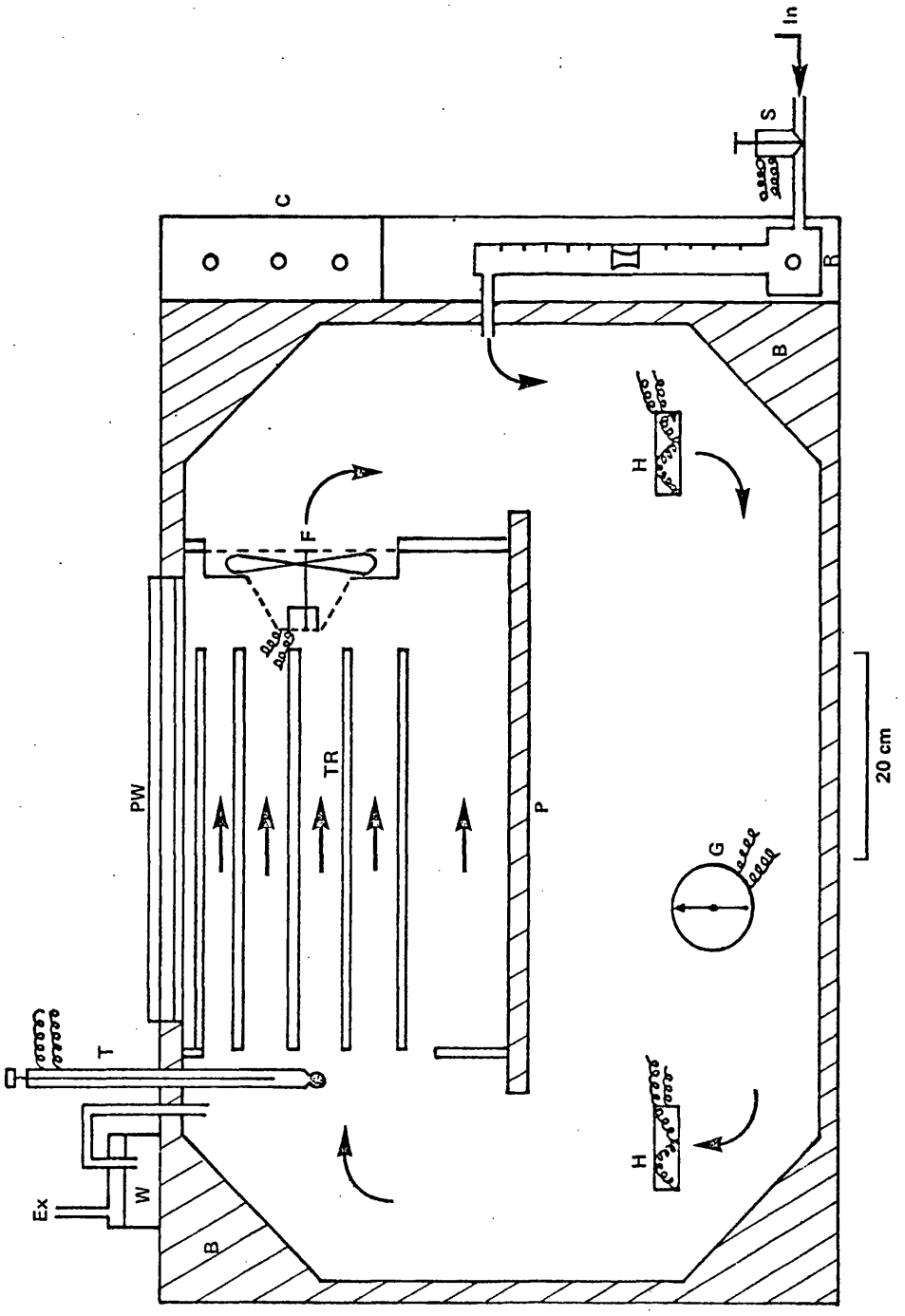


Figure 4 Schematic Cross Section of the Tray Drier

Key to figure 4

B	2.0cm blockboard lined with 0.6cm expanded polystyrene and aluminium foil.
C	Control box and master switches for fan, humidity controller and temperature controller
Ex	Air outlet
F	'Expelair' fan
G	Humidity gauge and photoelectric switching device
H	Heaters
In	Air Inlet
P	Central partition essentially converting the cabinet into an oval tunnel
PW	Double-glazed perspex window
R	Gas-flowmeter
S	Solenoid valve
T	Mercury-contact thermometer
TR	Drying trays
W	Water trap
	Direction of air flow ⟶

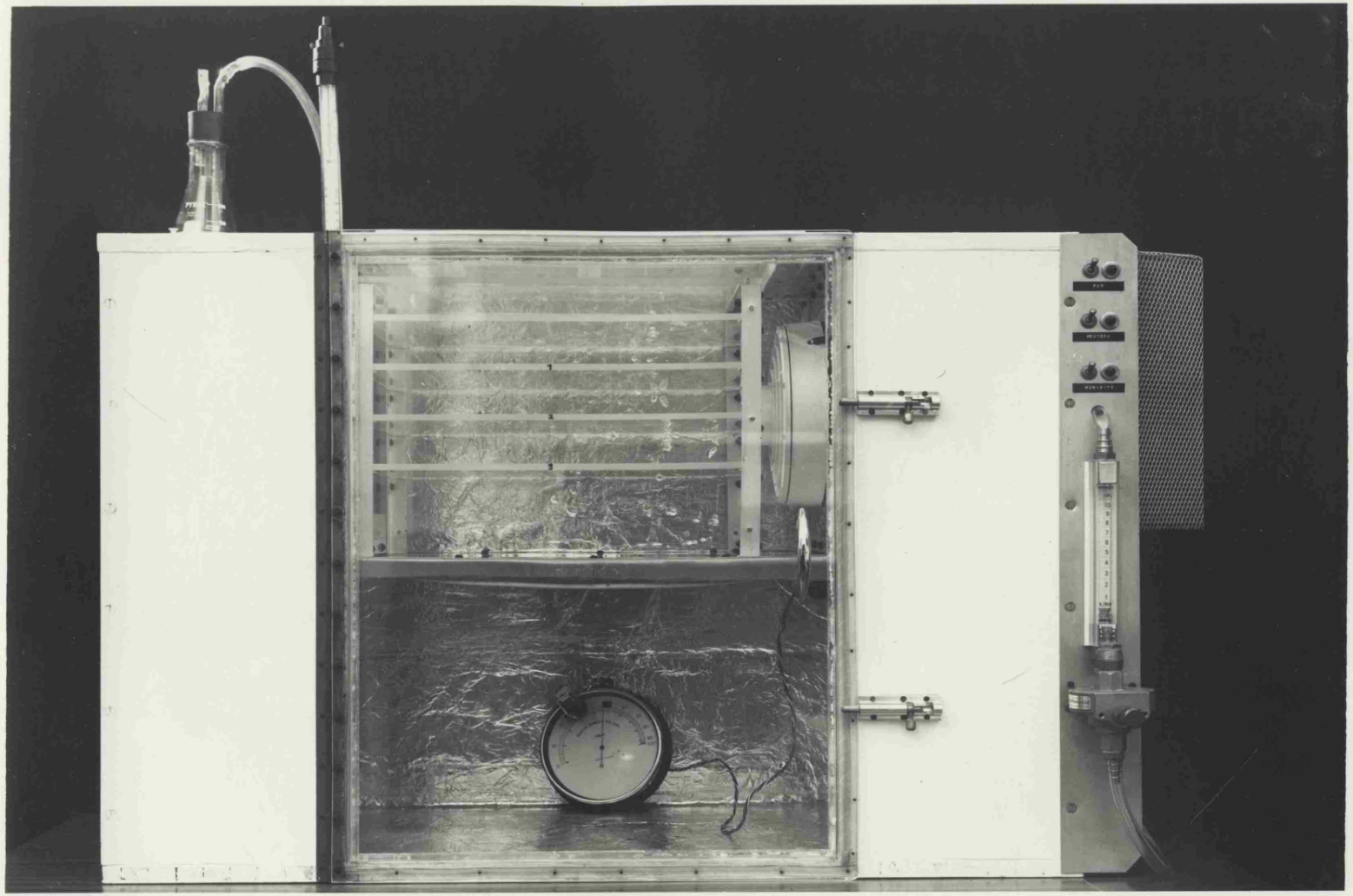
and plates 2 and 3). Air was circulated within the cabinet in the direction shown by a domestic 'Expelair' fan (type FXC6) with an appature of 15.3cm diameter and a gas flow-rate of 3.4 metres/sec.

Because of the high cost of commercially available units, temperature and humidity control circuits were designed and constructed in the University of Bath. Temperature control over the range 25 - 50°C ± 0.2°C was effected by a simple on/off switching circuit (employing Triac AO 1082 with trigger diode; Nobel Electronics, 5 - 7 High St, Welling, Kent), controlled by a mercury-contact thermometer (0 - 50°C; Ferris & Company Limited, Hillside Road, St. George, Bristol) and operating two greenhouse heaters, (Ecko Thermotube 60W, 240V, Figure 5).

Humidity control was effected by means of an on/off photo-electric switching circuit, regulated by a modified 'hair' hygrometer, and operating a solenoid valve which controlled the supply of relatively dry air to the cabinet (Figure 6). The hair hygrometer (0-100% relative humidity; Griffin and George, Ealing Rd, Alperton, Wembley, Middlesex) was modified so that, when the relative humidity (Rh) in the cabinet fell to a certain preset value, the indicator needle (extended by a small opaque flag 0.5cm x 0.5cm) passed between one of the photo-transistors (MRD 100) and the light source. This caused a voltage inbalance between the two inputs

PLATES 2 AND 3

THE DRYING CABINET


FAN
HEATER
HUMIDITY

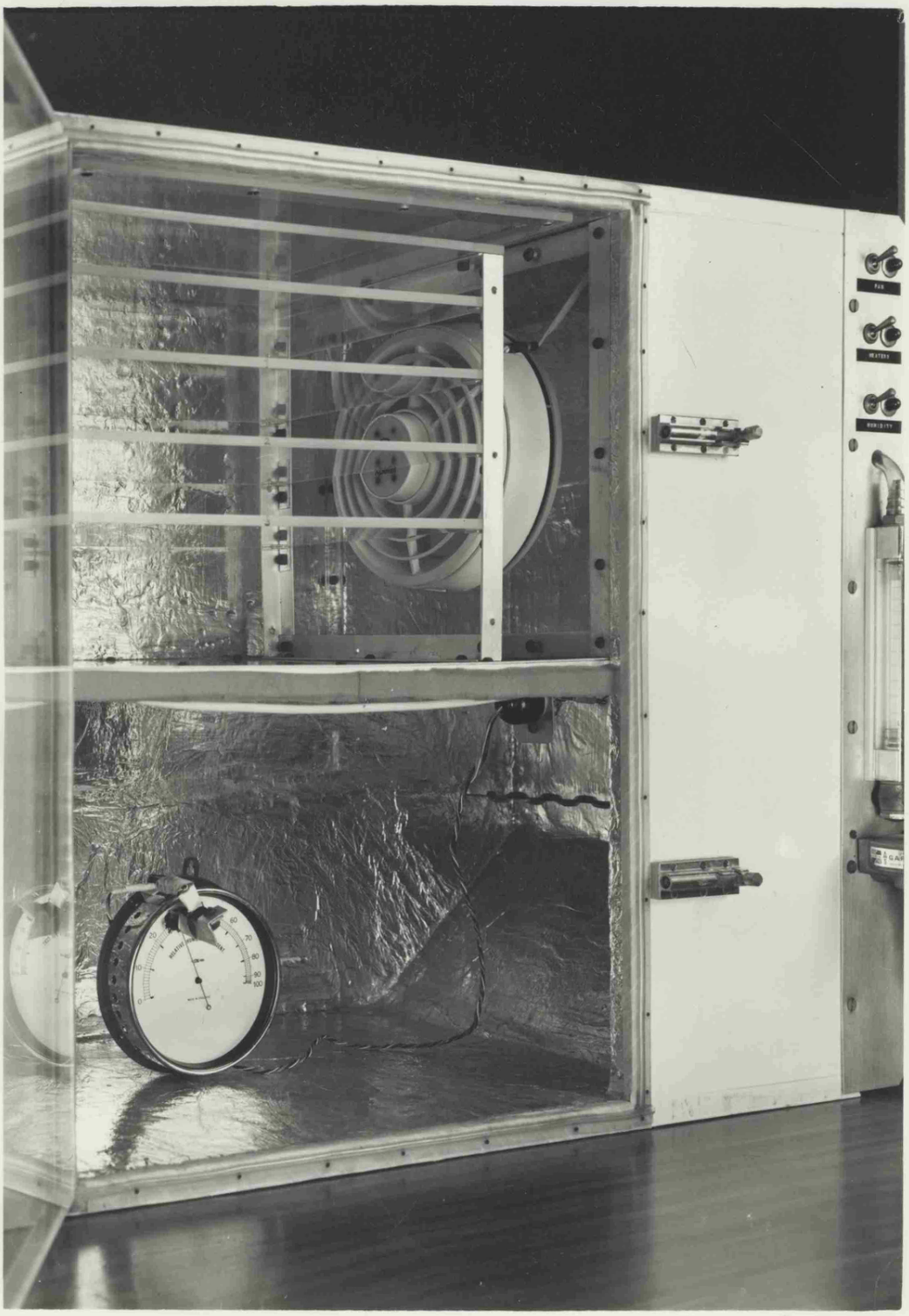


Figure 5 Temperature Controller Circuit Diagram

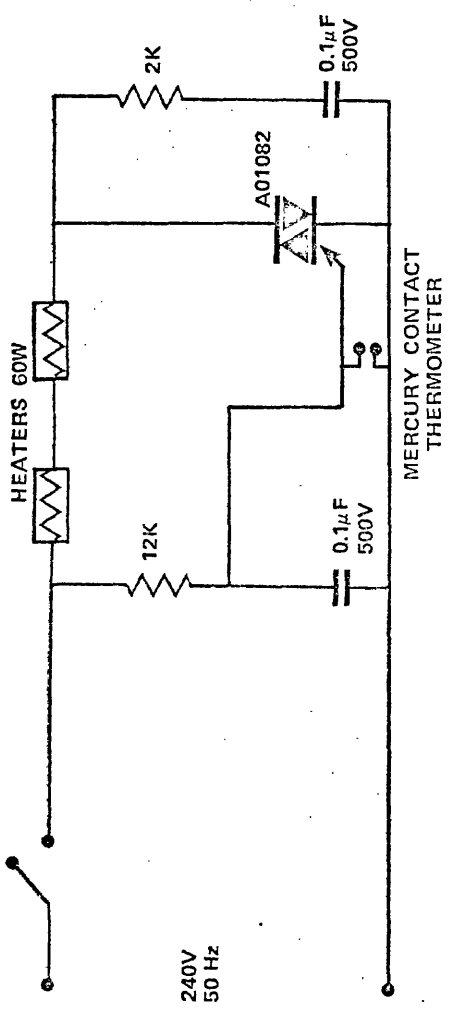
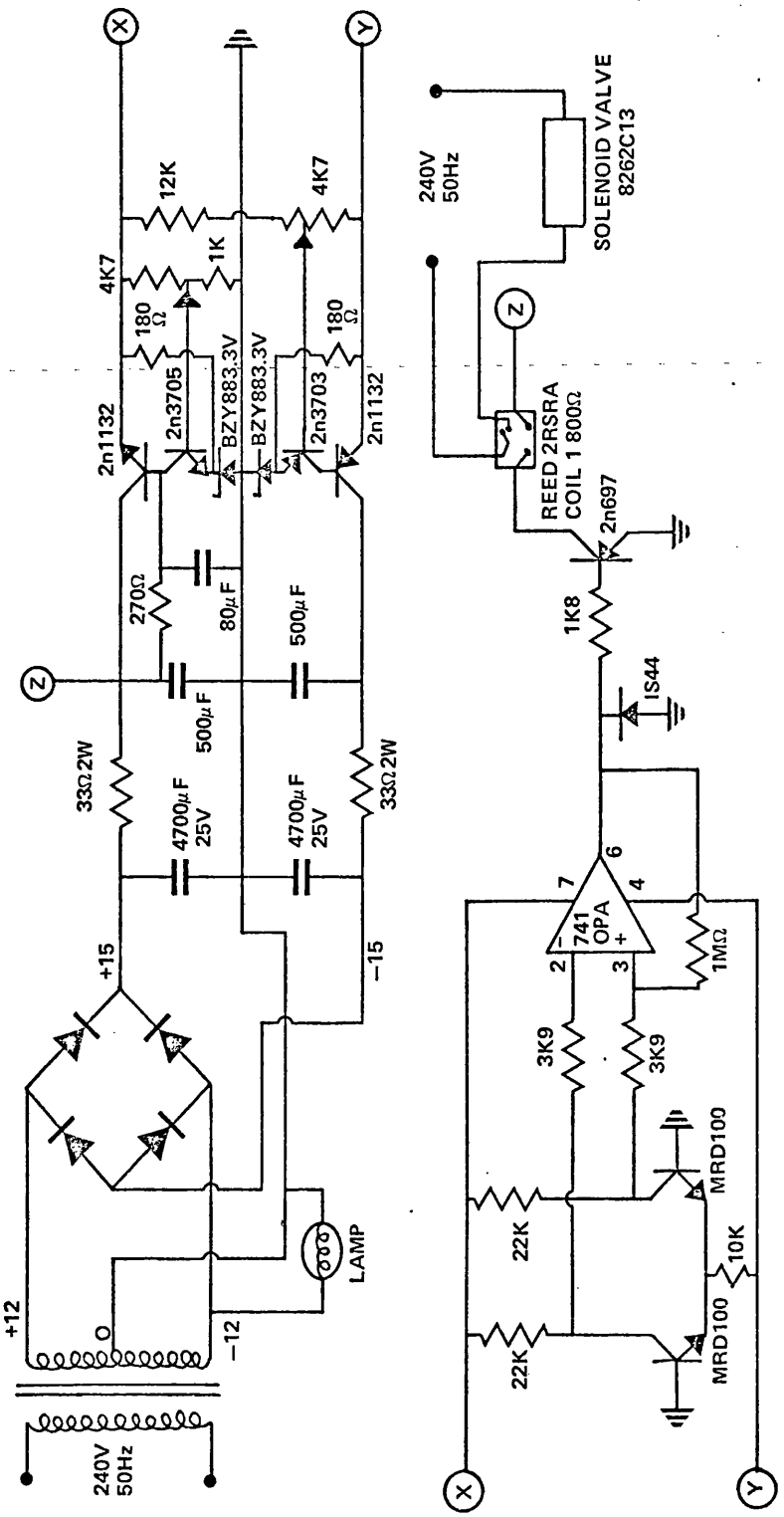


Figure 6 Humidity Controller Circuit Diagram



of the operating amplifier (741) which resulted in an output to the reed switch thus completing the 240V circuit to the solenoid valve (8262 C13; Dewrance Control Ltd., Grimrod Place, Skelsmersdale, Lancs.). The solenoid valve, which was normally in the 'open' position, then closed and the supply of air to the cabinet ceased. The air supply was taken from a 2.1×10^5 Pa compressed air line, and regulated into the cabinet via a gas flow meter with pressure regulator (Gapmeter GS-6F-1/10, Flowstat MNAM; G.A. Platon Ltd., Wella Rd, Basingstoke, Hampshire). Similarly, when the Rh value within the cabinet exceeded the predetermined value, the photo-electric controller resumed the supply of metered air into the cabinet.

The hair hygrometer, which had an entirely mechanical action, had a stated accuracy of $\pm$ 3% within the range 30 - 90% Rh. Routinely it was calibrated before use in an atmosphere of 40% Rh at 35°C in equilibrium with a saturated solution of potassium carbonate (O'Brien, 1948).

Procedure for the Drying of Yeast Cells

Samples of yeast (10 - 15g dry wt. equivalent) were suspended in a minimal volume of distilled water and filtered by vacuum through Whatman No. 44 paper to produce a thick paste of approximately 32% (w/w) dry matter. The pastes were extruded onto empty plastic Petri dishes in 0.2cm approx. diameter coils, using a modified plastic

disposable syringe (10ml) with the 'Luer' orifice sealed and replaced by five 0.2cm diameter holes.

Samples were placed on the trays within the drying cabinet and dried at 35°C by the recirculating air driven by the fan. Dry air was metered into the cabinet at a rate of 0.1 litre/min/g 'wet' yeast until the Rh value fell to 40% in the air circulating over the yeast. At this stage the humidity controller closed the dry air inlet and the cabinet was sealed from the atmosphere. The air within the cabinet stabilised at 40% Rh after 7 - 8h, and was in equilibrium with the yeast samples which were approximately 92% (w/w) dry matter. The ADY samples were then removed, for final equilibration of moisture content, and incubated for 36 - 48h at 20°C in an atmosphere of 32% Rh over saturated magnesium chloride solution (O'Brien, 1948), using an LEEC refrigerated incubator with forced air circulation. Samples of ADY were broken into short lengths (<3mm) and stored under nitrogen gas in sealed containers at 5°C.

RECONSTITUTION OF ACTIVE DRIED YEAST

Approximately 15ml of distilled water at 25°C or 38°C was added to a known weight (approximately 0.2g dry wt. equiv.) of ADY at the given temperature in a 20ml volumetric flask, and incubated statically for 10min. After shaking, for a further 5 mins and making up to

volume, the cells were dispersed thoroughly by gentle shaking.

ASSESSMENT OF THE FERMENTATIVE ACTIVITY OF YEAST SAMPLES

The fermentative activity of yeast samples was assessed by measuring the rate of release of carbon dioxide gas from a chemically defined medium using a Warburg apparatus. One unit of fermentative activity was defined as 1.0 μl of CO_2 gas released by 2mg dry wt. equiv. yeast after incubation at 30°C for 5 min.

Portions (200 μ litres) of yeast suspension were placed in the side arms of single side arm Warburg flasks (13ml capacity) using a constant delivery pipette. The main chambers of the Warburg flasks each contained 2.3ml chemically defined medium (modified from Atkin et al., 1945), having the following composition per litre of distilled water:

Glucose	36.0g
Asparagine	10.0g
$NaH_2PO_4.H_2O$	3.0g
$MgSO_4.7H_2O$	2.0g
KCl	0.8g
Thiamin HCl	5.0mg
Pyridoxine HCl	5.0mg
Nicotinic acid	50.0mg
Sodium citrate buffer M/3 pH 5.5	120.0ml

The Warburg flasks and manometers were assembled and flushed for 10 min with high-purity nitrogen gas (Air Products Ltd., Roath Dock, The Foreshore, Cardiff). The initial rate of carbon dioxide gas evolution was measured by standard manometric technique (Umbreit et al., 1964), at 30°C for a period of 40 - 50 min. The fermentative activity of each sample was taken as the mean of at least three determinations.

ANALYSIS OF YEAST LIPIDS

Lipid analysis of whole yeast cells was carried out on samples grown and harvested as previously described, but not subjected to drying. Yeast samples were freeze-dried (Model 30; Edwards High Vacuum Ltd, Manor Royal, Crawley, Sussex) for 24h and stored at -20°C in sealed containers. Plasma membrane preparations from 300mg dry wt. equiv. yeast were similarly stored, although the weight of membrane present was unknown due to the presence of large amounts of sucrose.

Extraction

Lipids were extracted from whole cells and plasma membrane preparations by a modification of the procedure used by Letters (1968b) Portions (200 - 350mg whole cells) of freeze- dried material were extracted with 15ml 80% (v/v) ethanol at 80°C for 15 min. The extract was then filtered through Whatman No 44 paper, the paper washed, and the extract stored at -20°C. The residue

was suspended in chloroform-methanol (1:1, v/v; 30ml) and stirred at room temperature (18 - 22°C) for 2h. The suspension was filtered and the residues re-extracted twice with chloroform-methanol (1:1, v/v; 30ml), each extraction lasted 3h at room temperature. The ethanol extract and three chloroform-methanol extracts were pooled and supplemented with 1ml chloroform containing butylated hydroxytoluene (0.005%, w/v) as an antioxidant (Neudoerffer and Lea, 1966) and stored at -20°C. The extract was filtered through a glass sinter (No 5) and evaporated to dryness *in vacuo* using a rotory evaporator (Büchi) and a rotory evapo-mix (Buchler).

Non-lipid material was removed from the extract by a modification of the procedure of Folch, *et al*. (1957). The extract was dissolved in chloroform-methanol (2:1, v/v; 5ml), 1ml of 0.118M KCl added, and the two phases were well mixed. The extract was centrifuged at 3,100*g* for two min to speed up separation of the two phases. The aqueous phase was drawn off and discarded, and the interface washed three times with the aqueous phase of chloroform-methanol-0.118M KCl (8:4:3, by volume; 1.5ml), to remove interfacial fluff.

Total Lipid Estimation

Total lipid was estimated by evaporating the purified extract to dryness *in vacuo* and redissolving in chloroform-methanol (2:1, v/v) and then making up to volume in a 5ml graduated flask. Small pans (1.0cm

diameter) were prepared from aluminium foil, heated to 110°C for 10 min, and then cooled in a vacuum desiccator containing silica gel and solid KOH (Rouser et al., 1967) and weighed; 100 µl of extract was added to each of three pans and the pans dried in vacuo to constant weight, in a vacuum desiccator.

Analysis of Phospholipids

Total phospholipid content of the lipid extract was determined by assaying the phosphorus content of a 25 µl portion of the extract using the method of Chen et al. (1956). Values for phosphorous content were converted to phospholipid by multiplying by 25 (which assumes an average molecular weight of 800 for phospholipid).

Individual phospholipids in the extract were separated by two-dimensional thin-layer chromatography using glass plates (20cm x 20cm) coated with a layer (0.4mm) of Kieselgel HF_{254} (Merck). Before use the plates were prewashed overnight in redistilled chloroform, to remove the ultraviolet-sensitive indicator, dried, and then activated by heating at 110°C for 1h. The plates were allowed to cool in the presence of a desiccant. The sample was applied to one corner of the plate and developed in the first direction with chloroform - Methanol-ammonia (0.88sp.gr.) (65:42:5:6.5, by volume) and in the second direction with chloroform-acetone-methanol-acetic acid-water (50:20:10:10:4, by volume). Both solvent systems contained butylated hydroxytoluene (0.005%, w/v). Phospholipid spots were detected by

exposing the plate to iodine vapour. Phospholipids were eluted from the silica gel by two volumes of chloroform-methanol-water (5:5:1, by volume; 3ml), followed by methanol (3ml), and finally methanol-acetic acid-water (95:1:5, by volume; 3ml). Samples were evaporated to a suitably small volume and after dilution (as appropriate) assayed for lipid phosphorus as already described.

The phospholipid fraction of whole cell lipid extracts was the base spot that remained on a Kieselgel $HF_{366+254}$ plate, after separation of the non-polar lipids (see below). The phospholipid fraction was eluted from the silica gel as described for the individual phospholipids.

Analysis of Non-Polar Lipids

Non-polar lipids were separated by quantitative thin-layer chromatography on glass plates (20 x 20cm) coated with a layer (0.4mm) of $Kieselgel_{366+254}$ (Merck). Each plate was divided into small 2.0cm channels as appropriate so that standard lipids could be run simultaneously with each unknown mixture. Plates were developed with petroleum spirit (40 - 60°C) - diethyl ether-acetic acid (70:30:2, by volume) containing butylated hydroxytoluene (0.005%, w/v), to a distance 18cm above the origin. Bands of lipid were located with an ultra-violet lamp and identified with simultaneously run. standards: Illumination at 254 nm was used for compounds containing unsaturated acyl residues, and 366 nm for

sterols and sterol esters.

The neutral-lipid fraction of whole-cell lipid extracts was taken to be the whole of the developed region of the silica-gel plate with the exception of the base spot (phospholipid fraction). Neutral-lipid fractions were extracted from the silica gel by elution with petroleum spirit (40 - 60°C; 50ml) followed by diethyl ether (50ml) and finally chloroform (50ml). Each eluate was removed in turn by filtration through a No 5 glass sintered filter.

Fatty acids were eluted from the silica gel with two portions (3ml) of diethyl ether-methanol (9:1, v/v) followed by two portions of chloroform (3ml) and assayed by the method of Heinen and de Vries (1966), and the fatty acid content calculated from a calibration curve prepared using oleic acid.

Sterols and sterol esters were eluted with three portions (3ml) of chloroform-methanol (4:1, v/v). The supernatants from elution of sterol esters were evaporated to dryness, redissolved in benzene (1.0ml) and supplemented with 4ml KOH (10%, w/v) in ethanol (90%, v/v). The mixture was refluxed on a steam bath for 2h after which time saponification was complete. After cooling, water (10ml) was added and the pH value of the saponification mixture adjusted to 1.0 with HCl. The saponification mixture was then extracted three times with diethyl ether (10ml) and the ether extracts dried over anhydrous Na_2SO_4, before being concentrated *in vacuo*. Sterols were

isolated by preparative thin-layer chromatography in petroleum spirit-diethyl ether-acetic acid (70:30:2, by volume) and the sterols eluted as already described.

Sterol contents were estimated using two methods; firstly by quantatative gas-liquid chromatography (See on) and secondly by a modification of the Liebermann-Burchard reaction described by Moore and Baumann (1952), in which contents of both fast- and slow-reacting sterols were calculated from a standard curve prepared using respectively ergosterol or cholesterol.

Gas-Liquid Chromatography

Samples were analysed using a Pye series 104 model 64 chromatograph with flame-ionisation detectors.

Methyl esters of fatty acids from total cell lipids, neutral lipids and phospholipids were prepared as follows: lipid extract containing 2 - 5mg of lipid was evaporated to dryness and dissolved in 0.5ml dry benzene and refluxed for 2h with 4ml methanolic HCl (5%, w/v; Lund and Bjerrum, 1931). A tube of anhydrous $CaCl_2$ was fitted to the condenser to maintain anhydrous conditions in the reaction vessel. After cooling, water (10ml) was added and the methyl esters extracted with diethyl ether (3 x 10ml). The ether extracts were dried over anhydrous Na_2SO_4, and isolated by preparative thin-layer chromatography using glass plates (20cm x 20cm) coated with a layer (0.4mm) of Kieselgel $HF_{366+254}$ (Merck), and developed with petroleum spirit (40-60°C)-diethyl ether-acetic acid (90:10:1,

by volume). The methyl esters were eluted from the silica gel with chloroform (3 x 3ml) and concentrated to a small volume (< 0.1ml). Separation of the methyl esters was carried out on 15% EGSS-X supported on 100-120 mesh Gas Chrom P packed in a 1.5m x 0.6cm stainless steel column, maintained at 175°C with a carrier gas (nitrogen) flow rate of 50ml/min and with the detector oven at 200°C. For determination of the total amounts of each fatty acid present in extracts, a known amount of internal standard (heptadecanoic acid) was added to the sample prior to methanolysis.

Sterols were prepared for gas-liquid chromatography by convertion to the appropriate acetate. Purified sterol (1mg approx.) was dissolved in dry pyridine (0.5ml) and acetic anhydride (0.5ml; Kuksis, 1967) and allowed to stand at room temperature (18 - 22°C) overnight. Excess reagent was evaporated off under a stream of nitrogen gas and the acetates takenup in a small volume (< 0.1ml) of chloroform. Sterol acetates were separated on 3% OV - 17 supported on 100 - 120 mesh Gas Chrom Q packed in a stainless steel column (2.6m x 0.6cm). The column was maintained at 255°C with a carrier gas (nitrogen) flow rate of 70ml/min, and the detector at 300°C. Sterol acetates were identified by their retension times relative to cholestane compared with similar values obtained from a standard solution of sterol.

ISOLATION OF PLASMA MEMBRANES

Preparation of Sphaeroplasts

Sphaeroplasts were prepared from freshly harvested cells by digestion with a glucanase from Basidiomycete QM 806, after pretreatment with 2-mercaptoethanol. The method was a modification of the method used by Alterthum and Rose (1973). Cells were washed twice in imidazole buffer (10mM; pH 6.0) containing sorbitol (1.4M) and $MgCl_2$ (10mM), and resuspended at 15mg dry wt. equiv./ml in buffered sorbitol-$MgCl_2$ solution supplemented with 2-mercaptoethanol (10mM). The reaction mixture (usually 20ml in 100ml conical flasks) was shaken for 15min at 30°C in a water bath at 100 rev./min. Cells were removed from the suspension by centrifuging at room temperature at 1,500g for 10 min, and washed twice in buffered sorbitol (1.4M) -$MgCl_2$ (10mM; pH 6.0).

Cells were resuspended in buffered sorbitol-$MgCl_2$ solution to the cell density stated previously, supplemented with Basidiomycete glucanase at a concentration of 300 units/ml (equivalent to 20 units/mg dry wt. equiv. cells) and incubated at 30°C. The addition of relatively large quantities of glucanase resulted in a final sorbitol concentration within the range 1.2 - 1.3M. Sphaeroplast formation was followed by adding portions (0.1ml) of suspension to 2.9ml of water or buffered sorbitol (1.4M) -$MgCl_2$ solution, gently shaking the diluted suspension. leaving at room temperature (18 - 22°C) for 10 min, and measuring the absorbance (E 600nm) of the suspension in

a Pye-Unicam SP 600 spectrophotometer. Sphaeroplast formation was judged to be complete when the absorbance of the diluted suspension in water was 25 - 30% of that at zero time. Completion of sphaeroplast formation was confirmed by microscopic examination.

Sphaeroplasts were removed from suspension by centrifuging at room temperature at 1,500g for 10 min, washed three times with imidazole buffer (10mM; pH 6.5) containing sorbitol (1.4M) and $MgCl_2$ (2mM) and resuspended in that buffer (20ml). The Basidiomycete glucanase was prepared, purified and assayed by the methods of Alterthum and Rose (1973).

Radioactive Labelling of Plasma Membranes

The plasma membranes of intact sphaeroplasts were labelled by iodination (^{125} I) of the tyrosine residues in membrane proteins, (Phillips and Morrison, 1970; Marchalonis et al., 1971) using a modification of the technique described by Shibeci et al. (1973). The reaction mixture (20ml) consisted of a suspension of sphaeroplasts (from 300mg dry wt. equiv. cells) in imidazole buffer (10mM; pH 6.5) containing sorbitol (1.4M), $MgCl_2$ (2mM), lactoperoxidase (10^{-7}M) and Na^{125} I (0.1 μCi/ml). Iodination was initiated by addition of H_2O_2 (8 μ moles in 0.1ml H_2O) and maintained by the addition of the same volume of H_2O_2 solution at one-minute intervals for a period of five minutes. Sphaeroplasts were removed by centrifugation as previously described, and washed six times with imidazole buffer (10mM; pH 6.5)

containing sorbitol (1.4M) and $MgCl_2$ (2mM) to remove excess Na ^{125}I.

Sphaeroplasts were lysed by a combination of osmotic and gentle mechanical shock, by resuspending in sucrose (8%, w/v; 3ml) and homogenising the suspension (20 strokes), using a Teflon hand homogeniser (0.1mm clearance).

Separation of Plasma Membranes by Isopycnic Density-Gradient Centrifugation

The sphaeroplast lysate was layered on the top of a discontinuous gradient of sucrose solutions, each of which was made up in tris buffer (10mM; pH 7.4). The gradient consisted of solutions containing 4ml of 10, 20, 30, 40, 50, 57.5, 62% (w/v) sucrose. An overlay of 2ml tris buffer (pH 7.4) was layered on the top of the tube. Tubes containing the gradients were centrifuged at 26,000g for 16h at 4°C. Visible bands in the gradient were removed with a hypodermic syringe and, after removing the remaining supernatant, the pellet was resuspended in water (3ml).

The concentration of sucrose in each fraction removed from the gradient was measured with a refractometer, and the readings converted to sucrose density by means of a standard curve using the data of de Duve et al. (1959). Portions (0.1 - 1.0ml), of each fraction from the gradient, of washings of the sphaeroplasts, and the sphaeroplast lysate, were added to 5ml of Unisolve

scintillation fluid in vials (20ml capacity), and the radioactivity of the contents of each vial determined by counting (for up to 20 min) in a Beckmann scintillation spectrometer (Model CPM 200; Beckmann Instruments, La Jolla, California, U.S.A.). Preparations of plasma membrane were freeze dried and stored at -20°C in sealed containers flushed with nitrogen gas.

OTHER ANALYTICAL METHODS

Protein

Protein was determined by the method of Lowrey et al. (1951). Standard curves were prepared relating extinction at 750nm to protein content per sample tube (within the range 25 - 500μg), using bovine sreum albumin (Sigma).

Yeast Dry Matter

Small pans (1.0cm diameter) were prepared from aluminium foil, preheated, cooled and weighed as described previously. Portions (200 - 300mg) of yeast sample were added to each of the three pans and the pans immediately reweighed. The pans were heated to 105°C in an oven until the yeast samples had been dried to a constant weight. The pans were cooled in the desiccator before being reweighed.

Total Nitrogen

Total nitrogen was assyed colourmetrically as ammonia (Searcy et al., 1967) following Kjeldahl digestion of

yeast samples (Bradstreet, 1965). Duplicate portions (approx. 30mg dry wt. equiv.) of yeast sample were digested with 2ml of digest reagent (five selenium-sodium sulphate crystal tablets (BDH) /100ml warm sulphuric acid), on a gas-fired microdigestion apparatus (Gallenkamp). After the reaction mixture had cleared, it was heated strongly for a further 20 min, and then allowed to cool. The digest solution was then washed into a beaker (100ml) stood in an ice bath, and the pH value brought to between 6.0 and 8.0 by addition of concentrated sodium hydroxide solution with constant stirring. Neutralisation is necessary for the assay of samples by the Searcy method (Frazer and Russell, 1969).

The neutralised digest was made up to 500ml and triplicate samples (0.5ml) were added to 1ml reagent A, the solutions mixed and then 1ml of reagent B was added. (Reagent A: Sodium salicylate 85g, sodium nitroprusside 0.6g made up to 1 litre with distilled water. Reagent B: Sodium dichloroisocyanurate 2.5g, made up to 1 litre with 0.3M NaOH). The tubes were incubated at 30°C for 10 min, 2.5ml of distilled water added, and the resulting emerald green colour was measured by reading the absorbance at 660nm using a reagent blank. Standard curves were prepared using appropriate ammonium sulphate solutions and water blanks, which had been run through the whole procedure.

Total Phosphorus

Portions (0.1ml) of yeast suspension in water (approx.

5mg dry wt. equiv./ml) were digested and assayed for phosphorus content, by the method of Chen et al. (1956). All glassware used was acid washed.

Glucose

Glucose was assayed using Boehringer Glucose Oxidase method (Boehringer Corporation Ltd., Uxbridge Rd, Ealing, London W.5.).

MATERIALS

Chemicals were supplied by the following manufacturers or agents:

Anderman and Company Limited, Battlebridge House, Tooley Street, London (Agents for E. Merck, Dormstadt.) supplied the Kieselgels HF_{254} and $HF_{366+254}$.

Field Instruments Limited, Richmond-upon-Thames, Surrey, England (Agents for Applied Science Laboratories Incorporated.) supplied standard mixtures of fatty-acid methyl esters and 15% EGSS-X on Gas Chrom P.

Koch-Light, Colnbrook, Buckinghamshire, England, supplied the N-Methyl-N-nitroso-N'-nitroguanidine (MNNG) and the butylated hydroxytoluene (BHT).

Phase Separations Limited, Queensferry, Flintshire, Wales, supplied the 3% OV - 17 on Gas Chrom Q.

The Radiochemical Centre, Amersham, Buckinghamshire, England, supplied the sodium (^{125} I) iodide.

Sigma Chemical Company Limited, Kingston-upon-Thames, Surrey, England, supplied the fatty acids (grade I), standard lipids, Tergitol NPX, Triton X - 100, spermine HCl, the bovine serum albumin, and the DL-α-tochopherol.

All other chemicals were analytical grade (i.e. 'AnalaR' or 'AR' or of the highest purity available commercially.

Chloroform, methanol and ethanol were redistilled before use. Pyridine was refluxed over barium oxide for 2h, redistilled and stored over potassium hydroxide. Benzene was stored and dried over sodium wire.

Before use, all glassware was soaked for at least 24h in 'Decon 75' (2%, v/v), rinsed twice in tap water and twice in glass-distilled water.

RESULTS

ISOLATION AND SELECTION OF MATING STRAINS FROM THE COMMERCIAL ADY STRAIN OF *Saccharomyces cerevisiae*

Vegetative cells of the commercial ADY strain were induced to form mature asci, the individual ascospores of which were isolated by microdissection following enzymic digestion of the ascan wall. The individual ascospores were cultured at 30°C to induce germination and growth of vegetative cells, which were then maintained at 5°C on slope cultures as a collection of potential mating strains.

Mating strains selected for further study showed haploid characteristics and had to satisfy the following criteria:

(a) The original ascospore isolate was obtained from an ascus containing four mature ascospores. Asci containing 1, 2 or 3 ascospores were not dissected.

(b) The vegetative cells demonstrated a specific mating reaction (i.e. conjunction with only one of the reference haploid mating strains, HQ5c 'α', HR8c 'a'; R.R. Powell personal communication).

(c) The vegetative cells, when incubated under conditions conducive to sporulation, were unable to form ascospores or asci.

(d) Vegetative cells inoculated at 3.0 - 4.0μg dry wt. equiv./ml into medium S (100ml in 250ml conical flasks incubated at

30°C in an orbital shaking incubator) grew at an exponential growth rate greater than 0.5 generation/h to a final cell yield in excess of 5.0mg dry wt. equiv./ml. Also that they were shown to be respiratory competant.

From a collection of 36 potential mating strains eight were found to satisfy the above criteria. Batches of ADY were produced from each of these strains using the growth and drying methods previously described in Methods. The fermentative activities of the yeast cells produced by each strain were determined both before and after drying (Table 1). Considerable variation was observed in the fermentative activities of the mating strains both before and after drying. Strains HP 44 and HP 87 showed little retention of fermentative activity after drying and, together with strains HP 41 and HP 72, were not considered for further study. Strain HP 92 exhibited the greatest retention of fermentative activity in the active dried form, although, the ADY produced from strain HP 95 proved to be the most active of all of the ADYs produced from the mating strains. It was considered that strains HP 92, HP 95 and HP 102 were mating strains suitable for use in the production of ADYs. They were subjected to mutagenisis treatment with MNNG and potential fatty-acid desaturase mutants isolated.

TABLE 1

FERMENTATIVE ACTIVITIES OF MATING STRAINS OF Saccharomyces cerevisiae BEFORE AND AFTER DRYING

Yeast mating strain	Fermentative activity ($\mu l CO_2$/5min/2mg dry wt. equiv. yeast) Fresh yeast	ADY	% Loss	% Dry Matter of ADY
HP 41*	-	-	-	-
HP 44	45.3	5.2	88.5	92.8
HP 54	42.4	25.1	40.8	89.4
HP 72*	-	-	-	-
HP 87	58.5	3.3	94.3	90.1
HP 92	37.2	31.9	14.2	91.7
HP 95	53.6	40.5	24.5	89.9
HP 102	48.4	33.7	30.3	90.1

* These strains gave a low yield from 1 litre fed-batch cultures. Cells would have had a high nitrogen and phosphorus content thus making them unsuitable for ADY production.

Several potential fatty-acid desaturase mutant strains were obtained that initially exhibited a requirment for Tween 80 (polyoxyethylene sorbitan mono-oleate; 2%, v/v) as a convenient source of unsaturated fatty acid; i.e. when streaked onto YEPD agar plates containing Tween 80, each strain produced isolated colonies up to 2mm in diameter after incubation at 30°C for two days. No growth was visible on unsupplemented medium after two days incubation; however, after prolonged incubation at 30°C for 5 days, small colonies with diameters within the range 0.1 - 1.0 mm were frequently observed. Only one unsaturated fatty-acid requiring, respiratory competant, strain (RP 108) was recovered after two subcultures on YEPD agar supplemented with Tween 80. The remaining strains were either unstable and failed to retain their auxotrophic character, or were shown to be respiratory deficient 'petites'.

The parent strain of the unsaturated fatty-acid auxotroph RP 108 was the mating strain HP 92. Both strains were mating type 'a' and respiratory competant. However, after prolonged incubation on unsupplemented YEPD medium colonies of strain RP 108 exhibited characteristics of a 'petite' or respiratory incompetant strain.

THE UNSATURATED FATTY-ACYL REQUIREMENT OF Saccharomyces cerevisiae MATING STRAIN RP 108

Growth on Solid Media

At an early stage in this study it was considered essential to confirm that the component of Tween 80 that promoted the growth of strain RP 108 was either a free fatty-acid or a fatty-acyl moiety of more complex molecule. Supplementation of solid medium (YEPD) with Tween 80 (2%, v/v) resulted in an increase in the size of colonies produced by individual cells of strain RP 108 spread on the surface of the medium (20 - 30 cells/8.5cm diameter petri dish) and incubated at 30°C for 5 days. No growth was visible on un-supplemented medium after only 2 days incubation.

The growth-promoting effect of Tween 80 could be similarly produced by supplementation of the basal medium with a long-chain unsaturated fatty-acid (7.1×10^{-5}M; Sigma, 99%). Saturated fatty acids of similar chain length did not promote growth of strain RP 108 on solid medium (Table 2). Oleic acid ($C_{18:1}\ \Delta^{9}\underline{cis}$) appeared to be the most effective growth-promoting acid of the unsaturated fatty acids used; however, assessing growth from colony size did not allow more precise comparisons to be made between the unsaturated fatty-acid supplements.

Growth of cells did occur on unsupplemented medium and on medium containing saturated fatty acids, and colonies

TABLE 2

GROWTH OF *Saccharomyces cerevisiae* MATING STRAIN RP 108 ON SOLID MEDIUM SUPPLEMENTED WITH TWEEN 80 AND VARIOUS FATTY ACIDS

Diameter (mm) of colonies after incubation at 30°C

Medium supplement*	2 days	5 days
None	< 0.1	0.1 - 1.0
Tween 80	0.1 - 1.5	2.0 - 5.0
$C_{16:0}$	< 0.1	0.1 - 1.0
$C_{16:1}\Delta^{9}$ cis	0.1 - 0.5	0.5 - 2.0
$C_{18:0}$	< 0.1	0.1 - 1.0
$C_{18:1}\Delta^{9}$ cis	0.1 - 0.5	1.0 - 4.0
$C_{18:2}\Delta^{9,12}$ all cis	0.1 - 1.0	0.1 - 2.0
$C_{18:3}\Delta^{9,12,15}$ all cis	0.1 - 0.5	0.5 - 2.0

* Concentrations of supplements in basal medium (YEPD):

Tween 80, 2% (v/v)

Fatty acids, 7.1×10^{-5}M

were visible after 3 days incubation. A random selection (approximately 20%) of these colonies were subcultured onto unsupplemented and supplemented ($C_{18:1}\Delta^{9}$ cis acid) media. Growth of all of these isolates was promoted by the addition of $C_{18:1}\Delta^{9}$cis fatty acid, indicating that they were unlikely to be composed of cells that had reverted to the wild type.

Further experiments indicated that an increase in the concentration of oleic acid in the medium from 10^{-4}M to 10^{-3}M did not increase the size of colonies produced. However, a decrease in fatty-acid concentration to 10^{-5}M and 10^{-6}M did considerably lower the mean colony size.

Experiments were also carried out to confirm that the only growth-promoting component of Tween 80 was an unsaturated fatty-acyl moiety and that the surface-active properties of Tween 80 were not significant in this study. Supplementation of solid media with Tween 60 (polyoxyethylene sorbitan monostearate; 2%, v/v) did not stimulate growth of strain RP 108. Analyses of the fatty-acyl residues of Tween 80 and Tween 60 (J.A. Hossack, personal communication.) have shown the major components to be $C_{18:1}$ (78.5%), $C_{16:1}$ (6.4%), $C_{16:0}$ (6.2%) in Tween 80, and $C_{18:0}$ (68.7%), $C_{16:0}$ (28.5%) with no detectable unsaturated fatty-acyl residues, in Tween 60.

Growth in Liquid Media

The fatty-acyl requirement of strain RP 108 was assessed from the exponential growth rates and yields of cells

from cultures supplemented with various fatty acids at concentrations between 10^{-5} and 2 x 10^{-3}M after 40 -50h incubation, compared with those values obtained from unsupplemented cultures (Figures 7 and 8). Supplementation of basal S medium with high concentrations (2 x 10^{-3}M) $C_{18:1}\Delta^{9}$ cis fatty acid (oleic) produced exponential growth rates and cell yields similar to those observed from cultures supplemented with Tween 80 (2%, v/v). Lower concentrations (5 x 10^{-4}, 10^{-4} and 10^{-5}M) of the $C_{18:1}$ acid produced progressively less stimulation of growth. Low concentrations (10^{-5} and 10^{-4}M) of the polyunsaturated fatty acids $C_{18:2}\Delta^{9,12}$ all cis (linoleic) and $C_{18:3}\Delta^{9,12,15}$ all cis (linolenic) increased the rate of growth of RP 108 cells to a similar extent as did similar concentrations of the $C_{18:1}$ acid, yet at high concentrations (5 x 10^{-4} and 2 x 10^{-3}M) these polyunsaturated fatty acids inhibited growth. Similarly, $C_{16:1}\Delta^{9}$ cis fatty acid at low concentrations (10^{-5} and 10^{-4}M) had no effect on growth of RP 108 cells, but at high concentrations (5 x 10^{-4} and 2 x 10^{-3}M) this fatty acid inhibited growth during incubation for 40h. All concentrations of the saturated fatty acids $C_{16:0}$ (palmitic) and $C_{18:0}$ (stearic) were unable to stimulate or inhibit growth of strain RP 108.

Promotion of growth of strain RP 108 by different concentrations of $C_{18:1}\Delta^{9}$ cis fatty acid is shown in Figure 9 . It is apparent that concentrations $\leq$ 5 x 10^{-5}M have little effect on growth. Exponential growth rate and cell yield are increased by a progressive increase in $C_{18:1}\Delta^{9}$ cis concentration, from 5 x 10^{-4} to 5 x 10^{-3}M.

FIGURE 7

EXPONENTIAL GROWTH RATES OF Saccharomyces cerevisiae MATING STRAIN RP 108 GROWN IN LIQUID MEDIUM SUPPLEMENTED WITH DIFFERENT FATTY ACIDS

Values given are either the means of duplicate determinations, or the mean values of between 4 and 11 determinations with the vertical bars indicating 95% confidence limits.

○ = Indicates no growth after 40h incubation.

● = Growth present, but not measured due to cloudiness caused by fatty-acid suspension.

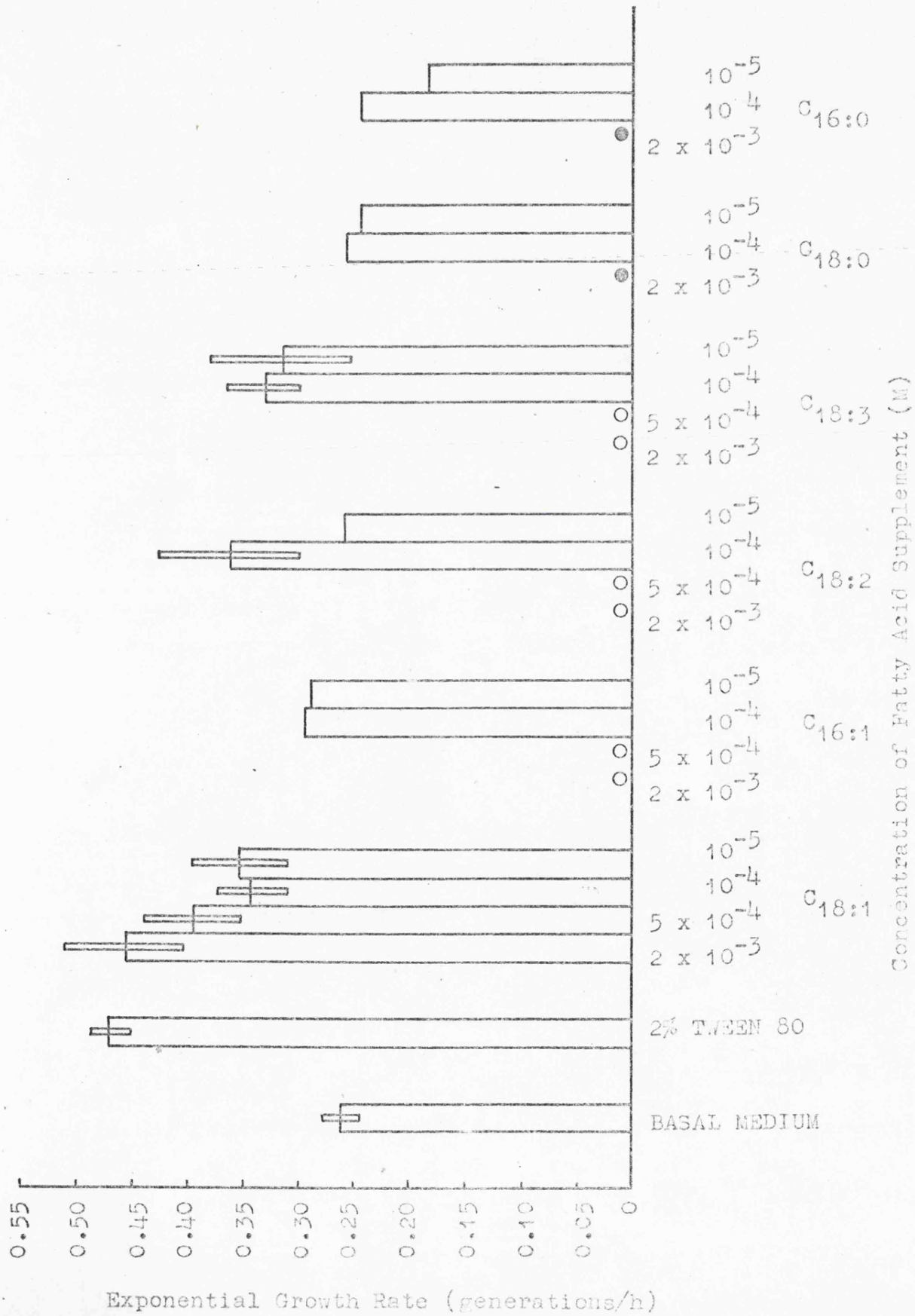

10^{-5}
10^{-4}
$C_{16:0}$
2×10^{-3}
10^{-5}
10^{-4}
$C_{18:0}$
2×10^{-3}
10^{-5}
10^{-4}
5×10^{-4}
$C_{18:3}$
2×10^{-3}
10^{-5}
10^{-4}
5×10^{-4}
$C_{18:2}$
2×10^{-3}
10^{-5}
10^{-4}
$C_{16:1}$
5×10^{-4}
2×10^{-3}
10^{-5}
10^{-4}
5×10^{-4}
$C_{18:1}$
2×10^{-3}
2% TWEEN 80
BASAL MEDIUM
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
Concentration of Fatty Acid Supplement (M)
Exponential Growth Rate (generations/h)

FIGURE 8

CELL YIELD OF Saccharomyces cerevisiae MATING STRAIN RP 108 GROWN IN LIQUID MEDIUM SUPPLEMENTED WITH DIFFERENT FATTY ACIDS.

Values given are either the means of duplicate determinations, or the mean values of between 4 and 12 determinations with the vertical bars indicating 95% confidence limits.

0 = Indicates no growth after 40h incubation.

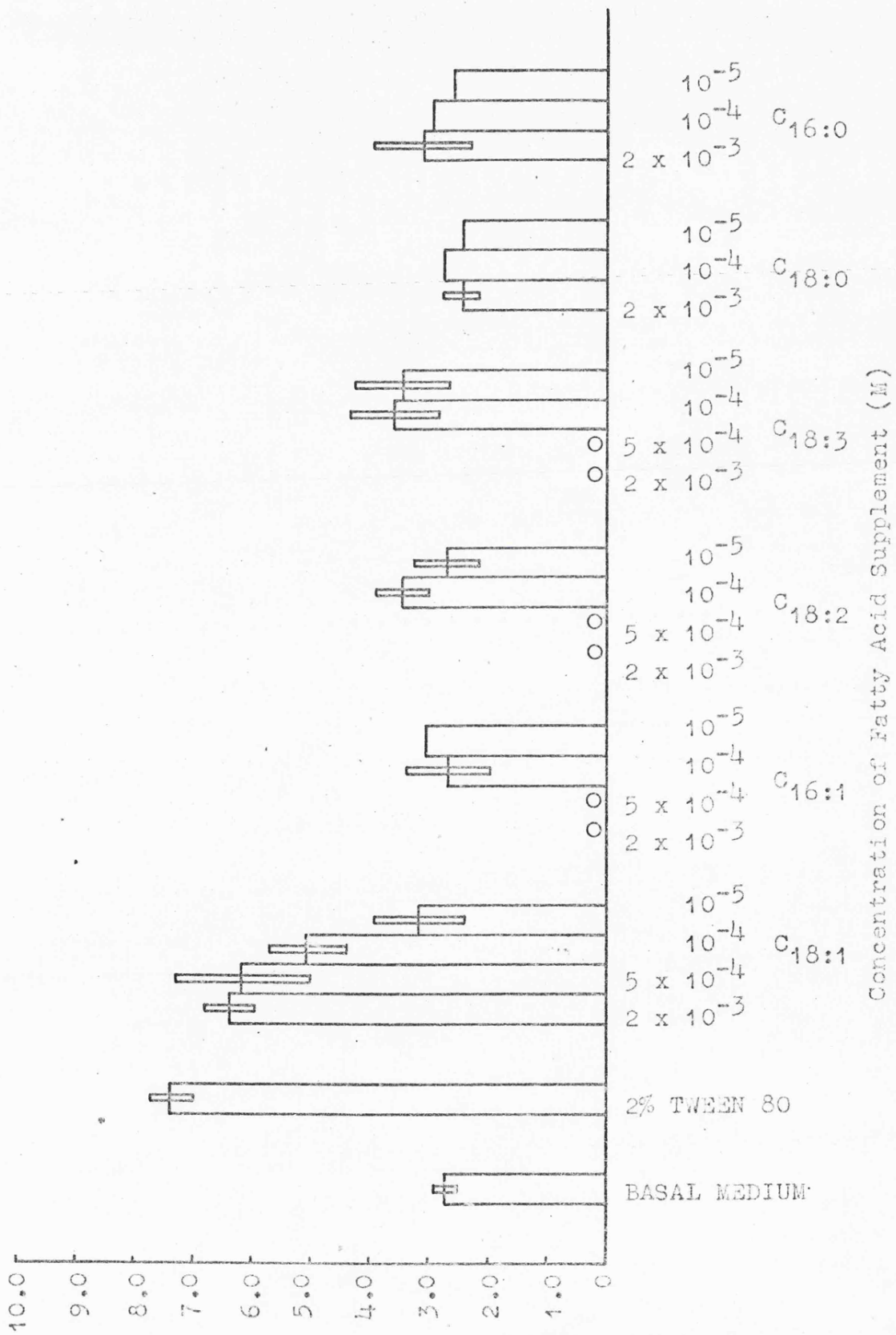
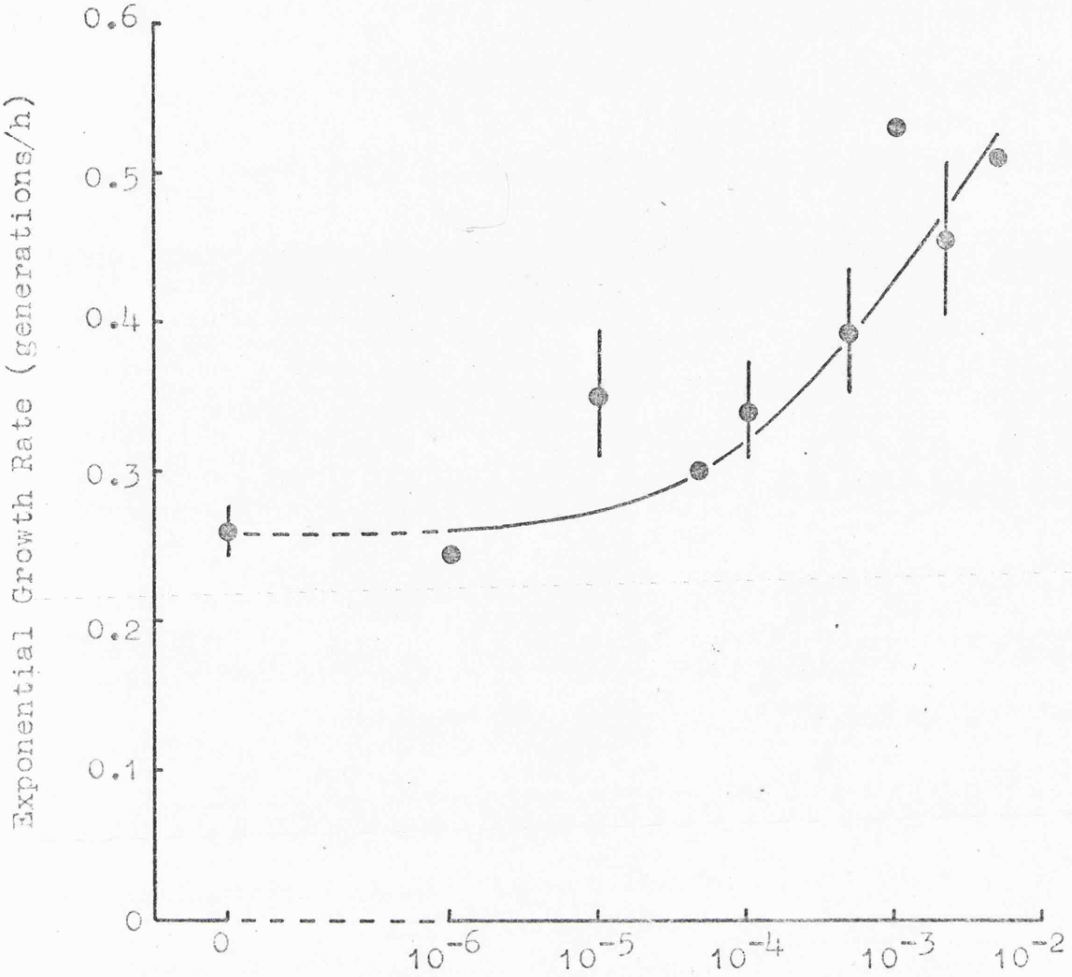
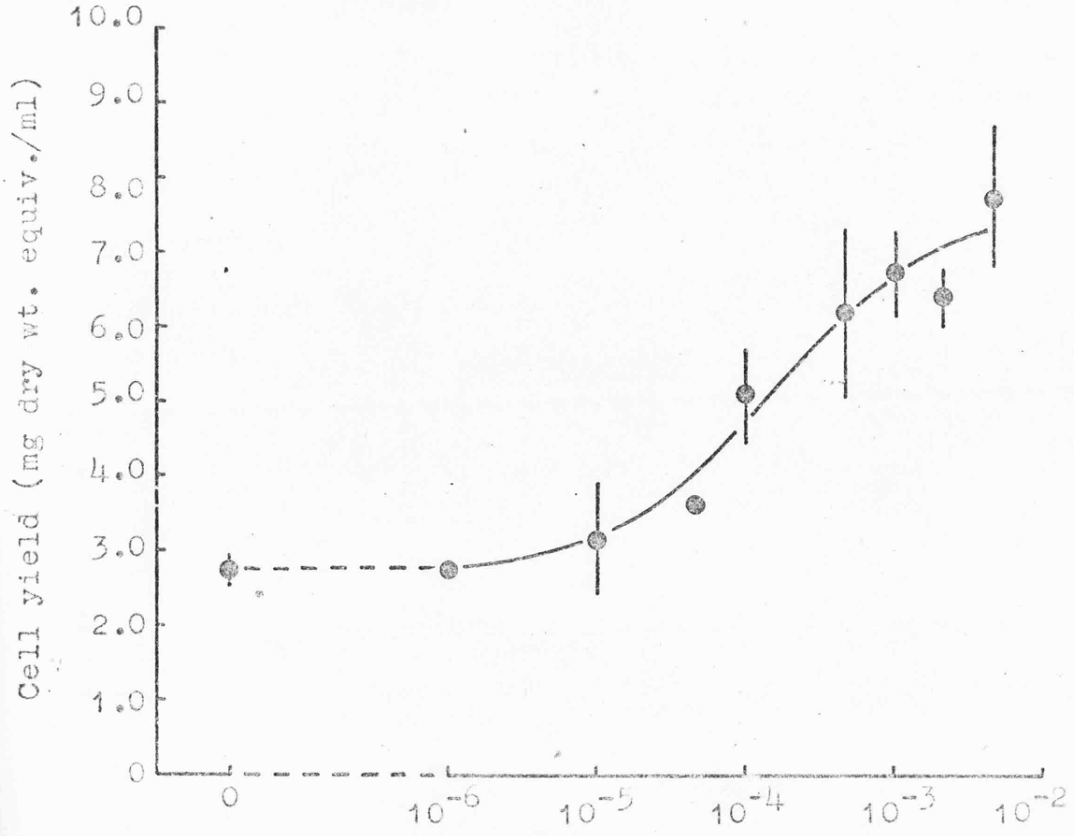

10^{-5}
10^{-4}
$C_{16:0}$
2×10^{-3}
10^{-5}
10^{-4}
$C_{18:0}$
2×10^{-3}
10^{-5}
10^{-4}
5×10^{-4}
$C_{18:3}$
2×10^{-3}
10^{-5}
10^{-4}
$C_{18:2}$
5×10^{-4}
2×10^{-3}
10^{-5}
10^{-4}
$C_{16:1}$
5×10^{-4}
2×10^{-3}
10^{-5}
10^{-4}
$C_{18:1}$
5×10^{-4}
2×10^{-3}
2% TWEEN 80
BASAL MEDIUM
10.0 9.0 8.0 7.0 6.0 5.0 4.0 3.0 2.0 1.0 0
Concentration of Fatty Acid Supplement (M)
Cell yield (mg dry wt. equiv./ml)

FIGURE 9

EXPONENTIAL GROWTH RATES AND CELL YIELDS OF *Saccharomyces cerevisiae* MATING STRAIN RP 108 GROWN IN LIQUID MEDIUM SUPPLEMENTED WITH DIFFERENT CONCENTRATIONS OF $C_{18:1}\Delta^{9}$ *cis* FATTY ACID

Values given are either the means of duplicate determinations, or the mean values of between 4 and 12 determinations with vertical bars indicating 95% confidence limits.


Exponential Growth Rate (generations/h)
0.6
0.5
0.4
0.3
0.2
0.1
0
0
10^{-6}
10^{-5}
10^{-4}
10^{-3}
10^{-2}


Cell yield (mg dry wt. equiv./ml)
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
0
10^{-6}
10^{-5}
10^{-4}
10^{-3}
10^{-2}
Concentration of $C_{18:1}$ Δ^9 cis (M)

However, no distinct maximal values were obtained. At a concentration of 5×10^{-3}M the dispersion of $C_{18:1}$ Δ^9 cis fatty acid was not uniform throughout the aqeous medium, and significant deposition of fatty acid was observed on the glass culture vessel. The maximal mean values for cell yield, and to a lesser extent exponential growth rate, of RP 108 cultures supplemented with high concentrations ($\geq 10^{-3}$M) $C_{18:1}$ Δ^9 cis, are nevertheless similar to those values obtained from cultures of strain HP 92 (RP 108 parent strain) grown in unsupplemented medium, i.e. a mean growth rate of 0.67 generations/h and a mean final cell yield of 7.5mg dry wt. equiv./ml.

Frequently, exponential growth in unsaturated fatty-acid supplemented cultures was only observed at low cell densities ($<$1.0mg dry wt. equiv./ml) above which the growth rate progressively decreased until a maximal cell density was reached. It therefore seemed likely that, even in media containing a high concentration of fatty acid, the growth rate of mutant yeast cells might have been restricted by the availability of the hydrophobic fatty acid. This postulate is particularly applicable to yeast cultures supplemented with high concentrations (2×10^{-3}M) of saturated fatty acids ($C_{16:0}$ and $C_{18:0}$) which were present in the liquid medium, not as a fine suspension of droplets, but as coarse suspensions of fatty-acid particles which formed large aggregations during incubation, thereby producing an inhomogeneous suspension. This phenomenon reflects

not only the hydrophobic nature of saturated fatty acids, but also their solid physical state at 30°C. Therefore, it was possible that saturated fatty acids were unable to promote growth of strain RP 108 due to their physical properties.

Attempts were made to solubilise saturated fatty acids ($C_{16:0}$, $C_{18:0}$; 10^{-3}M) in the aqeous growth medium by addition of the surface-active agents Triton X-100 and Tergitol NPX in concentrations of 0.1 and 1.0% (v/v). Both surface-active agents, at both concentrations, successfully dispersed large aggregates of saturated fatty acids in supplemented culture media, although solubilisation of the fatty acids was not complete. Triton X-100 (1.0 and 0.1%, v/v) was found to inhibit growth of strain RP 108 in basal medium (S) and in media supplemented with saturated or unsaturated fatty acids. Addition of Tergitol NPX (1.0 and 0.1%, v/v) to cultures of strain RP 108 resulted in low cell yields ($<$ 0.5mg dry wt. equiv./ml) when the culture was unsupplemented or supplemented with a saturated fatty acid ($C_{16:0}$, $C_{18:0}$; 2×10^{-3}M). However, addition of Tergitol to cultures supplemented with unsaturated fatty acid ($C_{18:1}$ Δ^{9} cis, $C_{18:2}$ $\Delta^{9,12}$ all cis)did not adversely effect growth, but similarly did not significantly increase the growth rates or the cell yields of such cultures.

From these observations one may conclude that cells of strain RP 108 are more susceptible to damage by surface-

active agents when the content of unsaturated fatty acids in their cell lipids and lipid-containing organelles is restricted. Also, although addition of Tergitol NPX to a fatty acid suspension considerably increases the availability of both saturated and unsaturated fatty acids to cells in aqueous suspension, the lack of any growth-promoting affect by this improved availability indicates that saturated fatty acids are unable to promote growth of strain RP 108 for reasons other than those of unfavourable physical properties. Due to the possible deleterious affects of surface-active agents on the cells of strain RP 108 grown for ADY production, fatty acid supplements used in subsequent experiments were not solubilised in the aqueous growth medium.

Specificity of Requirement of Strain RP 108 for Unsaturated Fatty Acids

Having shown that growth of strain RP 108 was promoted by supplementation of the basal medium with $C_{18:1}\ \Delta^{9}\underline{cis}$ fatty acid, experiments were carried out to determine whether growth was promoted by, (a) $C_{18:1}$ fatty acids with different sites of unsaturation, and (b) mono-unsaturated fatty acids of various chain lengths.

Cultures of strain RP 108 were supplemented with $C_{18:1}$ fatty acids (10^{-3}M) that were structural isomers of $C_{18:1}\ \Delta^{9}\underline{cis}$ (oleic) i.e. $C_{18:1}\ \Delta^{6}\underline{cis}$ (petroselinic), $C_{18:1}\ \Delta^{9}\underline{trans}$ (elaidic) and $C_{13:1}\ \Delta^{11}\underline{cis}$ (vaccenic). Similarly cultures were supplemented with mono-unsaturated

fatty acids (10^{-3}M) of various chain lengths; i.e. $C_{14:1}\Delta^{9}$cis (myristoleic), $C_{16:1}\Delta^{9}$cis (palmitoleic) and $C_{20:1}\Delta^{11}$cis (eicosaenoic). The last fatty acid is of interest because the site of unsaturation is a similar distance from the methyl end of the molecule (the so called ω position) to that of the unsaturation site found in $C_{18:1}\Delta^{9}$cis.

The inhibitory effect of $C_{16:1}\Delta^{9}$cis fatty acid has already been referred to (Figures 7 and 8) and is discussed more fully later in this thesis. With this exception, all of the fatty acids referred to above were unable to effect an increase or decrease in the exponential growth rate of strain RP 108 when compared with those values obtained from unsupplemented cultures. Supplementation with the mono-unsaturated fatty acids C_{14} and C_{20} acids did not result in an increased cell yield when compared with unsupplemented cultures. However, supplementation with the mono-unsaturated structural isomers of $C_{18:1}\Delta^{9}$cis fatty acid (Δ^{6}cis, Δ^{11}cis, Δ^{9}trans), although producing a comparable cell yield after 40h to that obtained from unsupplemented cultures (2.5 - 2.9mg dry wt. equiv./ml), gave a much higher cell yield (5.2 - 6.0mg dry wt. equiv./ml) after prolonged incubation at 30°C for up to 90h. It is possible that cells of strain RP 108 routinely subcultured on medium containing $C_{18:1}\Delta^{9}$cis fatty acid (as Tween 80) require a period of adjustment before they are able to incorporate different, yet closely related, fatty acids and subsequently achieve their maximal growth.

Growth Inhibitory Effect of High Concentrations ($>10^{-4}$M) of $C_{16:1}$ Δ^9 cis and $C_{18:2}$ $\Delta^{9,12}$ all cis Fatty Acids on Strain RP 108.

While not apparently relevant to a study of active dried yeast, these observations were considered worthy of further study for the following reasons: (a) $C_{16:1}$ Δ^9 cis fatty acid is known to be one of the major naturally occurring fatty acids in yeasts. Inhibition of yeast growth by supplementation with this fatty acid was therefore a surprising observation. (b) Growth inhibition of strain RP 108 by high concentrations of the polyunsaturated fatty acids $C_{18:2}$ $\Delta^{9,12}$ all cis and $C_{18:3}$ $\Delta^{9,12,15}$ all cis prevented addition of these supplements to one-litre fed-batch cultures where an initial fatty-acid concentration $> 10^{-3}$M was required. Active dried yeasts enriched with these fatty acids could not therefore, have been prepared.

In a study of the desaturase yeast mutant KD 115, Wisnieski et al. (1970) reported similar inhibition of cell growth by high concentrations of fatty acids, notably $C_{18:2}$ $\Delta^{9,12}$ all cis. They concluded that certain fatty acids were growth inhibitory in high concentrations. However, more recent studies have shown that $C_{18:2}$ $\Delta^{9,12}$ all cis and $C_{18:3}$ $\Delta^{9,12,15}$ all cis fatty acids are extremely susceptible to oxidation by air at temperatures $> 0^{o}C$. The hydroperoxides formed are, inhibitory to the growth of Escherichia coli (Gamage et al., 1971), inhibitory to enzyme systems (Matsushita et al., 1970; Gamage and Matsushita, 1972),

damaging to cytochrome systems (Desai and Tappel, 1963) and disruptive to microsomal membranes (Bidlack and Tappel, 1973). As aerobic yeast cultures incubated at 30°C appeared to provide suitable conditions for hydroperoxidation of unsaturated fatty acids, it seemed most probable that inhibition of growth of strain RP 108 by high concentrations of polyunsaturated fatty acids was due to an accumulation of toxic oxidation products.

The initial reaction in the peroxidation of linoleic acid results in the formation of a conjugated diene which has a characteristic absorption peak at 233nm. This may be used to estimate the degree of peroxidation in lipid preparations containing linoleic acid (Dahle et al., 1962; Privett and Blank, 1962). Aqueous suspensions (100ml) containing $C_{18:2}\ \Delta^{9,12}$ all cis and $C_{18:3}\ \Delta^{9,12,15}$ all cis fatty acids (2×10^{-3}M) were incubated at 30°C as previously described for small-scale growth cultures. Aliquots (1ml) were removed from the fatty-acid suspension and added to 2ml methanol which solubilised the fatty acid droplets. Samples were scanned in 1cm silica cuvettes in a Pye-Unicam SP 1800 recording spectrophotometer. It was not possible to conduct similar experiments using the yeast growth medium S as components of this medium strongly absorbed ultra-violet light. The results of these experiments are shown in Figures 10 and 11.

The $C_{18:2}$ acid had a characteristic absorbance between 204 and 207 nm with little evidence of hydroperoxidation

FIGURES 10 AND 11

CHANGES IN ULTRA-VIOLET ABSORPTION SPECTRA OF SUSPENSIONS OF $C_{18:2}\,\Delta^{9,12}$ all cis (Figure 10) AND $C_{18:3}\,\Delta^{9,12,15}$ all cis (Figure 11) FATTY ACIDS INCUBATED IN DISTILLED WATER AT 30°C.

Numbers given in the figures indicate incubation time (h) relating to individual traces.

FIGURE 10

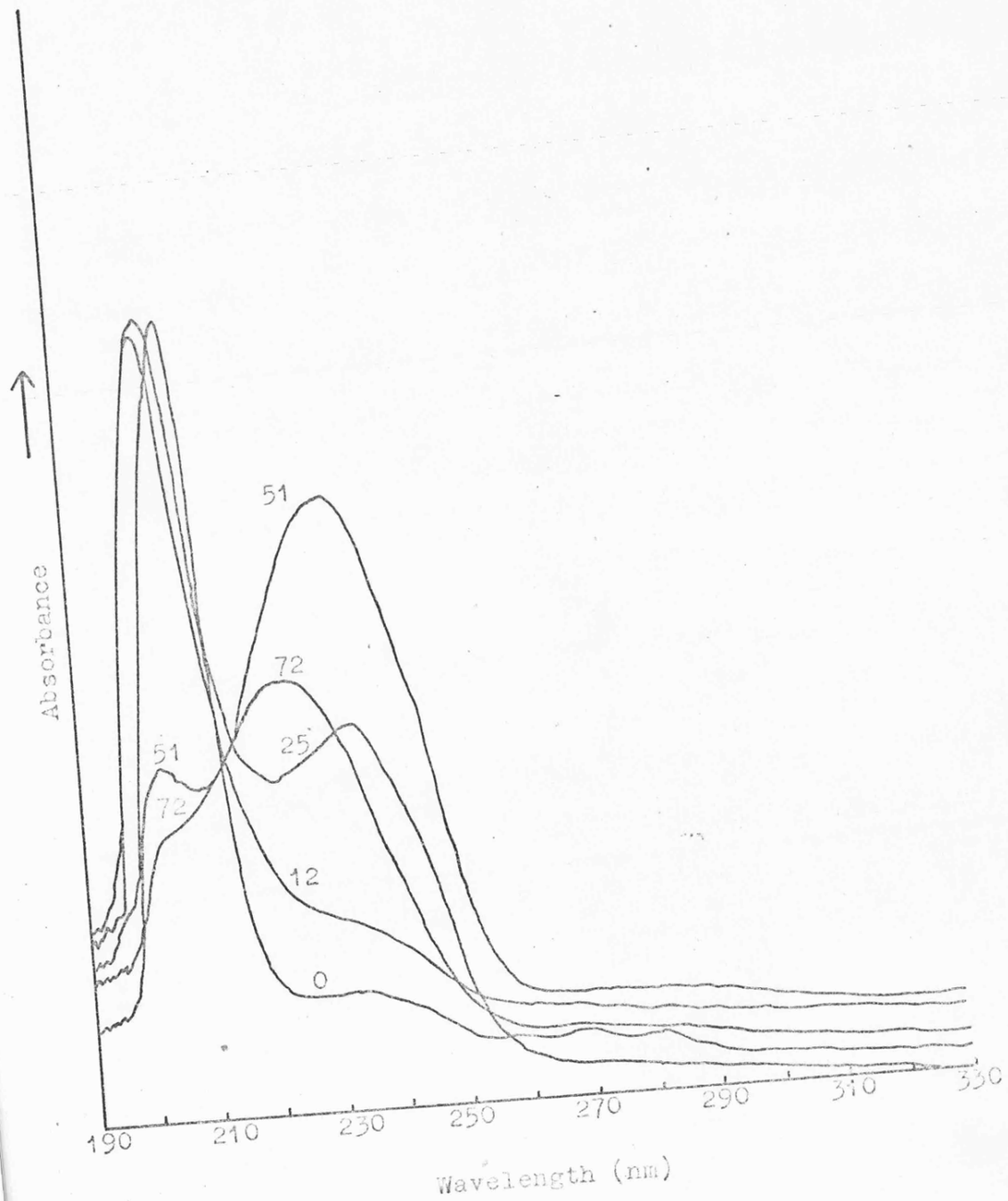
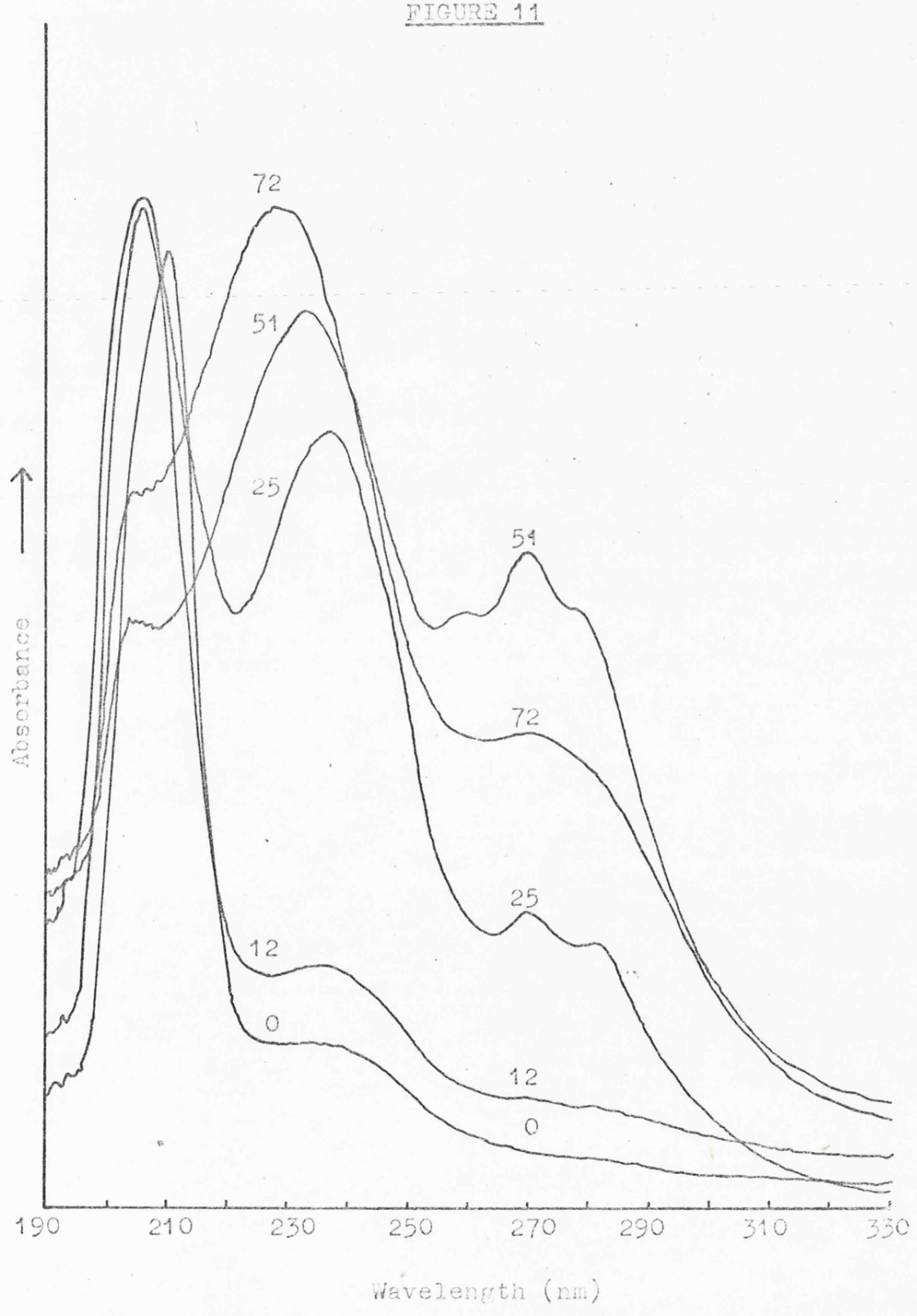

Absorbance
51
72
51
25
72
12
0
190
210
230
250
270
290
310
330
Wavelength (nm)

FIGURE 11


72
51
25
51
72
25
12
0
12
0
Absorbance
190
210
230
250
270
290
310
330
Wavelength (nm)

prior to aerobic incubation. During aerobic incubation at 30°C the proportion of the $C_{18:2}$ acid in the suspension diminished, and a secondary component was detected with an absorption maximum within the range 235 - 224 nm. These data correspond well with the reported absorption maximum of 233 nm for 9-hydroperoxylinoleic acid. A diminution of the quantity of this component after 72h incubation was probably due to further breakdown to short-chain molecules as proposed by Dominguez and Canales (1974). Similar results were obtained from the aerobic incubation of the $C_{18:3}$ acid indicating diene conjugation, except that a third component was detected with an absorption maximum of 270 nm.

These results indicate that significant hydroperoxidation of polyunsaturated fatty acids occurred in the small-scale (100ml) culture system employed in this study. Indeed, peroxidation was likely to have progressed at higher rates in yeast culture medium S due to the catalytic properties of ferric ions, sugars and low pH values (5.5; Lundberg and Jarvi, 1968).

Attempts were made to prevent peroxidation of $C_{18:2}$ $\Delta^{9,12}$ all <u>cis</u> (2×10^{-3}M) in small-scale cultures of strain RP 108 by the addition of 0.01% (w/v) butylated hydroxytoluene (BHT) or 0.01% (w/v) DL - α - tochopherol as anti-oxidants. Cultures were incubated as previously described for periods up to 50h. No growth occurred in the $C_{18:2}$ supplemented cultures whereas unsupplemented

cultures and cultures supplemented with $C_{18:1}\Delta^{9}$ cis had reached stationary phase. When cultures were incubated for 160h non-logerithmic growth occurred in the presence of $C_{18:2}$ fatty acid after an initial lag phase of approximately 60h, provided that one of the anti-oxidants was present. At no time during this study was growth of strain RP 108 observed in media supplemented with $C_{18:2}$ fatty acid without the presence of an anti-oxidant, indicating that formation of toxic hydroperoxides were partially responsible for the lack of growth in $C_{18:2}\Delta^{9,12}$ all cis fatty acid-supplemented media.

The cells that had grown after 160h incubation in the $C_{18:2}$- supplemented medium containing anti-oxidant were subcultured into identical media. After a lag phase of 10-15h, growth commenced and a peak cell density within the range 7.0 - 8.5mg dry wt. equiv./ml acheived after 60 - 100h incubation. The cells were harvested and found to exhibit a requirement for $C_{18:1}\Delta^{9}$ cis and $C_{18:2}\Delta^{9,12}$ all cis fatty acids on solid and in liquid medium. Revertants were not detected.

One may conclude that cells of strain RP 108 routinely subcultured on medium supplemented with one unsaturated fatty acid ($C_{18:1}\Delta^{9}$ cis) are unable immediately to utilise different unsaturated fatty acids (such as $C_{18:2}\Delta^{9,12}$ all cis) without a period of metabolic adjustment, i.e. at first they exhibit an abnormally long lag phase and non-logarithmic growth, but after

one or two passages are able to grow typically in media supplemented with the 'new' fatty acid.

Similar experiments were carried out, as already outined, using $C_{16:1}$ Δ^9 cis fatty acid. Cells of strain RP 108 behaved similarly when transferred to the $C_{16:1}$-supplemented medium, exhibiting at first an extended lag phase (>60h) followed by more typical growth, after 1 - 2 passages, similar to that observed in cultures supplemented with $C_{18:1}$ Δ^9 cis fatty acid. Anti-oxidants were not required, however, for growth of strain RP 108 in $C_{16:1}$ Δ^9 cis-supplemented medium. Cells of strain RP 108 that had been conditioned to grow on media supplemented with either the $C_{16:1}$ or $C_{18:2}$ acid were maintained for further study on agar slopes similarly supplemented.

Incorporation of Saturated Fatty Acids into Strain RP 108

Although it had been demonstrated conclusively that growth of strain RP 108 could only be promoted by supplementation with an unsaturated fatty acid, it was considered conceivable that, under certain conditions of unsaturated fatty-acid supplementation, saturated fatty acids might also be incorporated into the cells. If true, this phenomenon would prove a useful additional method by which the fatty-acyl composition of yeast cells might be altered.

Cells of strain RP 108 were grown to stationary phase

in small scale (100ml) cultures, as previously described, supplemented with different proportions of unsaturated ($C_{18:1}\Delta^9$ cis) and saturated fatty acids ($C_{16:0}$, $C_{18:0}$, $C_{14:0}$ and $C_{20:0}$). The final total fatty acid concentration in each medium was 2×10^{-3}M. Cells from each culture were harvested and their fatty-acyl compositions determined (Table 3) using the methods previously described. The cell yields from all cultures supplemented with $C_{18:1}\Delta^9$ cis fatty acid were within the range 4.8 - 5.6mg dry wt. equiv./ml. However, the yield from unsupplemented cultures was within the range 1.9 - 2.2 mg dry wt. equiv./ml.

Perhaps the most important observation made during these experiments was that, although growth of strain RP 108 is promoted by supplementation with $C_{18:1}\Delta^9$ cis fatty acid, the strain does not possess an obligate requirement for such supplementation. Presumably sufficient quantities of $C_{16:1}$ and $C_{18:1}$ acids are produced endogenously by the cells, so permitting a low level of growth in medium S, i.e. the strain RP 108 is 'leaky'.

Supplementation of cultures of strain RP 108 with $C_{18:1}\Delta^9$ cis fatty acid (2×10^{-3}M) resulted in incorporation of that fatty acid into cell lipids. Endogenous production of $C_{16:1}$ fatty-acyl residues was suppressed. Significant quantities of $C_{16:0}$ fatty acid were incorporated into cells of strain RP 108 in

TABLE 3

FATTY-ACYL COMPOSITION OF CELLS OF Saccharomyces cerevisiae MATING STRAIN RP 108 GROWN IN MEDIA SUPPLEMENTED WITH DIFFERENT PROPORTIONS OF UNSATURATED AND SATURATED FATTY ACIDS TO A FINAL CONCENTRATION OF 2×10^{-3}M

Figures given are mg/100mg dry wt. cells.

(1) and (2) refer to data from two separate experiments.

t = Trace

- = Not detected

FATTY ACID SUPPLEMENT (2×10^{-3}M)

Fatty-acyl residue	Unsupplemented 1	Unsupplemented 2	$C_{18:1}$ 100% 1	$C_{18:1}$ 100% 2	$C_{18:1}$ 70% $C_{16:0}$ 30% 1	$C_{18:1}$ 70% $C_{16:0}$ 30% 2	$C_{18:1}$ 50% $C_{16:0}$ 50% 2	$C_{18:1}$ 30% $C_{16:0}$ 70% 2	$C_{18:1}$ 70% $C_{18:0}$ 30% 1	$C_{18:1}$ 30% $C_{18:0}$ 70% 2	$C_{18:1}$ 70% $C_{14:0}$ 30% 2	$C_{18:1}$ 70% $C_{20:0}$ 30% 2
$C_{10:0}$	t	t	t	t	t	t	t	t	t	t	t	t
$C_{12:0}$	t	t	t	t	t	t	t	t	t	t	t	t
$C_{14:0}$	t	t	t	t	t	t	t	t	t	t	0.05	t
$C_{15:0}$	-	t	-	t	t	t	t	-	t	t	t	t
$C_{16:0}$	0.36	0.41	0.24	0.54	0.44	0.73	0.68	0.85	0.23	0.30	0.80	0.30
$C_{16:1}$	0.37	0.34	-	-	0.62	0.56	0.79	0.94	t	0.27	0.86	0.12
$C_{18:0}$	0.39	0.46	0.31	0.35	0.22	0.28	0.22	t	0.32	0.37	0.26	0.31
$C_{18:1}$	1.16	1.46	2.99	2.65	2.58	2.19	1.48	1.04	2.93	1.82	1.73	2.58
$C_{18:2}$	t	-	-	-	-	-	-	-	-	-	-	-
$C_{20:0}$	-	-	-	-	-	-	-	-	-	-	-	0.48
TOTAL	2.31	2.70	3.54	3.58	3.85	3.77	3.20	2.87	3.47	2.80	3.72	3.81
% Unsaturated	66.1	66.5	84.5	74.0	83.0	72.8	71.0	68.9	84.4	74.6	69.5	70.8
Free Fatty Acid	-	-	-	-	-	-	0.17	0.39	-	0.45	-	0.33

cultures supplemented with a smaller concentration of the $C_{18:1}$ fatty acid, although a proportion of the $C_{16:0}$ residues was probably present as free fatty acid attached to cell walls or in intracellular 'pools', and not incorporated into cell lipids. $C_{18:0}$ and $C_{20:0}$ fatty acids were not significantly incorporated into cell lipids, but were probably present within the cell lipid extracts as free fatty acids. Similarly, $C_{14:0}$ fatty acid was not apparently incorporated into cell lipids although an appreciable increase in the content of $C_{16:0}$ residues within these cells was observed. It was possible that some $C_{14:0}$ fatty acid was converted into $C_{16:0}$ residues (Erwin, 1973b).

DEVELOPMENT OF A ONE-LITRE BATCH CULTURE SYSTEM WITH NUTRIENT FEEDS FOR PRODUCTION OF YEASTS SUITABLE FOR CONVERSION TO ACTIVE DRIED YEAST

Preliminary experiments were carried out to produce small quantities of yeast for ADY production in conventional batch cultures without nutrient feed. Portions (1 litre) of a 1:24 dilution of medium G in distilled water supplemented with ammonium sulphate 0.15% (w/v) and mono-ammonium phosphate 0.0133% (w/v) (equivalent to a one-third dilution approx. of the final fed-batch culture medium) were sterilsed by membrane filtration, (0.45 μm, Oxoid), inoculated with 0.1mg dry wt. equiv. Parent strain cells / ml and

incubated at 30°C for 24h. The yield from these cultures was poor (< 3 g dry wt./l) although the fermentative activity of the fresh yeasts was high (50 - 66 μl CO_2/5 min/2mg dry wt. equiv.). After drying, less than 5% of the initial fermentative activity was recovered which indicated that the growth method was unsuitable for production of yeast for conversion to ADY.

The batch system with linear feeds devised for production of yeasts suitable for convertion to ADY (see Methods), employed media formulated for use in a system that utilised an exponential nutrient feed. The feed was composed of components of both media G and N. The duration of nutrient feed G which regulated cell growth was standardised at 24h for the Parent strain, together with an inoculum of 1.0 $\pm$ 0.1 mg dry wt. equiv. yeast/ml (in 500ml St medium).

Preliminary experiments indicated that a considerable lowering of the percentage loss in fermentative activity was achieved by feeding medium N to the culture only during the first 12h incubation. Additionally, the concentration of ammonium sulphate in medium N that was fed to the culture, was lowered from 4.5g to 3.375g/ 250ml to produce yeasts with a relatively low nitrogen content. Further decreasing the concentration of ammonium sulphate to 2.255g/250ml produced a low yield of low nitrogen yeast which lost a greater proportion of fermentative activity during drying (Table 4).

TABLE 4

GROWTH OF Saccharomyces cerevisiae PARENT STRAIN IN ONE-LITRE CULTURE SYSTEM WITH VARIOUS NITROGEN AND OTHER NUTRIENT FEEDS

Duration of feed (h)		Weight Ammonium Sulphate in medium N (g)	Cell Yield g dry wt. equiv./ litre	% Nitrogen in cells	Fermentative Activity $\mu l CO_2$/5min/2mg dry wt.equiv. yeast			% Dry Matter of ADY
Medium G	Medium N				Fresh yeast	ADY	% Loss	
24	24	4.50	15.5	8.65	70.2	20.0	71.5	91.0
24	18	4.50	13.1	8.45	63.9	31.2	51.1	90.0
24	12	4.50	12.8	8.85	48.2	28.2	45.5	90.2
24	24	3.375	11.0	7.83	70.7	10.9	84.6	92.5
24*	12*	3.375*	15.2	7.52	53.2	42.9	19.4	92.2
24	12	2.255	10.8	6.90	45.6	29.1	36.2	91.8

* Indicates optimal conditions

During these experiments the quantity of sodium bicarbonate added to the culture with medium G, was lowered proportionally from 7.5g (for 4.5g addition of ammonium sulphate) thus maintaining a pH value at 4.5 - 6.0 within the culture. Sodium bicarbonate addition was required to neutralise excess sulphate ions that would otherwise have produced an unacceptable lowering of the pH value of the medium as ammonium ions were metabolised by the yeast.

The growth system required further modification when attempts were made to grow mating strains HP 92 and RP 108 using the feed conditions outlined above. Due to their intrinsic lower growth rates, inocula (1.0 ± 0.1 mg dry wt. equiv. /ml) of these strains were unable to metabolise the nutrient feeds at a sufficient rate to maintain an acceptable pH value (4.5 - 6.0). Sodium bicarbonate excess to the requirements of the culture caused a rise in the pH value (> 7.0) which subsequently inhibited growth. To overcome this problem, and avoid the complication of slower feed rates during the early stages of growth, the overall culture feed time was increased to 36h (feed of medium G) and medium N was fed during the first 18h of incubation. In addition, the final yields of cells from cultures of the mating strains were lower than the yields obtained from cultures of the Parent strain. To enable production of yeasts with comparable nitrogen contents, the quantity of ammonium sulphate fed to these cultures was further

lowered from 3.375g to 2.875g. These data are summarised in Table 5.

Figure 12 shows the changes in medium composition and cell concentration of a typical fed-batch culture of the Parent strain utilising the cultural conditions itemised in Table 5. Cell growth was linear after 4 - 5h incubation, while the pH value of the culture was maintained between 4.5 - 6.0. Moreover, the cells were grown under conditions of absolute nitrogen depletion for the final three hours of incubation. A similar pattern of results was obtained from cultures of mating strain HP 92 except that the cell yield after 36h incubation was within the range 10.3 $\pm$ 0.3g dry wt. equiv./litre.

EFFECT OF FATTY-ACID SUPPLEMENTATION OF *Saccharomyces cerevisiae* MATING STRAIN RP 108 ON RECOVERY OF FERMENTATIVE ACTIVITY AFTER DRYING

Batches of yeast strain RP 108 were grown in one-litre cultures supplemented with various concentrations of unsaturated fatty acid, and one saturated fatty acid. The yeasts were harvested, converted to ADYs, and their fermentative activities determined both before and after drying. Two temperatures were chosen for the reconstitution of ADYs, namely, 38°C the optimum temperature for the commercially produced ADY and, 25°C

TABLE 5

CELL YIELDS, TOTAL NITROGEN AND PHOSPHORUS CONTENTS, OF *Saccharomyces cerevisiae* PARENT AND MATING STRAINS GROWN IN THEIR RESPECTIVE OPTIMISED ONE-LITRE CULTURE SYSTEMS

YEAST STRAIN	DURATION OF FEED (h)		QUANTITY FED TO CULTURE (g)		CELL YIELD* (g dry wt. equiv./litre)	% NITROGEN OF* CELLS	% PHOSPHORUS OF* CELLS
	Medium G	Medium N	Nitrogen	Phosphorus			
PARENT	24	12	0.716	0.108	16.0±0.93	7.30±0.32	0.900±0.05
MATING STRAIN HP 92	36	18	0.610	0.108	10.7±0.34	6.51±0.31	1.01±0.12
MATING STRAIN RP 108 (Grown with various fatty-acid supplements)	36	18	0.610	0.108	10.3±0.61	6.96±0.13	0.952±0.03

*Values given are the means of at least five replicates ± 95% confidence limits

FIGURE 12

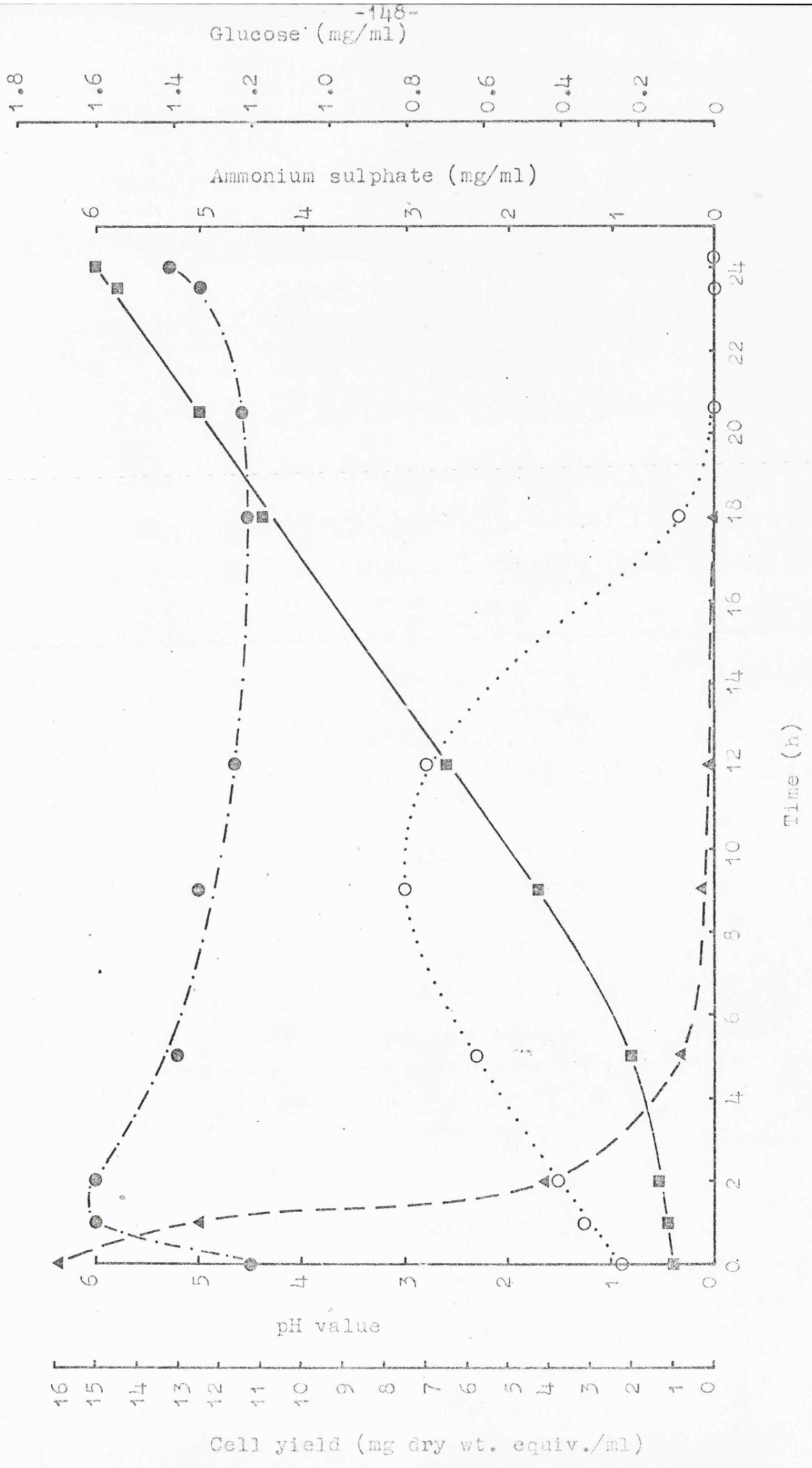
CHANGES IN MEDIUM COMPOSITION AND CELL CONCENTRATION OF A FED-BATCH CULTURE OF *Saccharomyces cerevisiae* PARENT STRAIN

■———■ Indicates cell yield

●–·–·–● Indicates pH value

○·····○ Indicates concentration of ammonium sulphate

▲– – – –▲ Indicates concentration of glucose


Glucose (mg/ml)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Ammonium sulphate (mg/ml)
6
5
4
3
2
1
0
0
2
4
6
8
10
12
14
16
18
20
22
24
Time (h)
pH value
6
5
4
3
2
1
0
Cell yield (mg dry wt. equiv./ml)
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0

a suboptimal reconstitution temperature. The fatty-acid supplementes employed during this study were:

(a) $C_{18:1}\Delta^{9}$ cis 1000mg/litre
(b) $C_{18:1}\Delta^{9}$ cis 350mg/litre
(c) $C_{18:1}\Delta^{9}$ cis 150mg/litre
(d) $C_{18:1}\Delta^{9}$ cis 150mg/litre
plus $C_{16:0}$ 300mg/litre
(e) $C_{18:2}\Delta^{9,12}$ all cis 350mg/litre
(plus 0.01% BHT)

Analysis of the fatty-acid composition of the spent medium and yeast water washes combined indicated that only approximately 100mg of a supplement of 1000mg of the $C_{18:1}$ acid was not absorbed by the yeast. Supplementation with higher concentrations (1,500mg/litre) of the $C_{18:1}$ fatty acid resulted in a greater proportion of fatty acid remaining in the spent medium. Subsequent analysis of the fatty-acyl residues of whole cell extracts of these yeasts indicated that the proportion of fatty acid absorbed was less than 90% (approximately 70%). An explaination of this discrepancy was provided by the observation that a hydrophobic deposit containing yeast cells was observed on the glass components of the culture system. This deposit was probably fatty acid which was not subsequently detected in the spent medium.

A concentration of 150mg of the $C_{18:1}$ acid/litre was found to be the lowest level of supplementation that gave cell yields comparable with the Mating Parent strain HP 92 (approximately 10g dry wt. equiv./litre). Attempts to grow batches of strain RP 108 in media containing less than 150mg $C_{18:1}$ fatty acid/litre were unsatisfactory due to poor cell yields. A concentration of 350mg/litre for both $C_{18:1}$ Δ^{9} cis and $C_{18:2}$ $\Delta^{9,12}$ all cis fatty acids was chosen as a suitable intermediate value.

The most suitable concentration of $C_{16:0}$ fatty acid (300mg/litre) in the mixtures containing both unsaturated and saturated fatty acids was calculated to be just in excess of the maximum quantity removed by the cells (as already described). Subsequent analysis of the fatty-acyl residues in cells grown under such conditions indicated that approximately 60 - 70% of the 300mg of $C_{16:0}$ was removed by the cells.

The fermentative activities of yeasts grown in media supplemented with various fatty acids are presented in Table 6 . Data obtained from batches of the non-auxotrophic Mating Parent strain (HP 92) and the Parent strain are provided for comparison. The mean fermentative activities of strains HP 92 and RP 108 grown in media supplemented with various fatty acids were similar (within the range 36.3 - 43.2 $\mu l CO_2$/5 min/2mg dry wt. equiv.) but were considerably lower than the mean value

TABLE 6

EFFECT OF DRYING ON THE FERMENTATIVE ACTIVITY OF Saccharomyces cerevisiae MATING STRAIN RP 108 GROWN IN FATTY-ACID SUPPLEMENTED MEDIA

FERMENTATIVE ACTIVITY $\mu l/CO_2/5min/2mg$ dry wt. equiv.

YEAST STRAIN	RP 108.	RP 108	RP 108	RP 108	RP 108	HP 92 (MATING PARENT)	PARENT
FATTY-ACID SUPPLEMENT	$C_{18:1}$	$C_{18:1}$	$C_{18:1}$	$C_{18:1}$, $C_{16:0}$	$C_{18:2}$	NONE	NONE
mg/litre	1000	350	150	150 300	350	NONE	NONE
FRESH YEAST	38.6±2.2	36.3±1.7	37.0±3.2	36.3±2.1	43.2±4.8	39.6±4.3	57.3±4.9
ADY (Reconstituted at 38°C)	30.4±2.2	28.6±2.7	22.5±3.6	22.9±2.4	28.9±3.5	34.6±4.2	48.3±6.2
% loss	21.6±4.5	21.3±6.1	39.3±7.4	36.6±8.4	33.2±5.6	12.6±1.9	17.2±5.5
ADY (Reconstituted at 25°C)	25.4±1.7	23.8±2.0	19.4±1.5	19.0±2.9	17.4±2.3	31.7±6.1	N/D
% loss	34.3±4.9	34.4±4.8	47.4±3.4	47.5±6.6	59.7±4.0	20.2±7.4	N/D
% DRY MATTER OF ADY	92.4±0.3	92.3±0.4	92.4±0.5	92.2±0.7	92.2±0.2	92.4±0.8	92.5±0.8

Values given are the means of at least five replicates showing 95% confidence limits

N/D = Not determined

obtained for fresh Parent yeasts (57.3). After reconstitution at 38°C, the Mating Parent strain (HP 92) showed a similar proportional retention of fermentative activity to that obtained from the Parent strain. The mating strain RP 108, exhibited different proportional retention of fermentative activity when the dried yeast was reconstituted at 38°C, depending on the nature and concentration of the fatty-acid supplement used. The highest proportional retention of fermentative activity was observed in ADYs produced from yeast grown in media supplemented with 1000 and 350mg $C_{18:1}\Delta^{9}$ cis fatty acid/litre. There appeared to be little difference between the values obtained. Both the fermentative activities and the proportional retention of activity of both of these ADYs were lower than the values obtained for strain HP 92.

When the concentration of the $C_{18:1}$ fatty acid supplement was lowered to 150mg/litre, the ADYs produced were less active and the proportional retention of fermentative activity after reconstitution at 38°C was considerably diminished. The results obtained for ADYs produced from yeast grown in media supplemented with 300mg $C_{16:0}$/litre in addition to 150mg $C_{18:1}\Delta^{9}$ cis/litre were similar.

The mean fermentative activity of ADYs produced from yeasts supplemented with 350mg $C_{18:2}\Delta^{9,12}$ all cis/litre was similar to that value obtained from ADYs derived from yeasts grown in media supplemented with a similar

quantity of the $C_{18:1}$ fatty acid. However, results calculated as proportional loss of fermentative activity indicated that the $C_{18:2}$ fatty-acid supplemented ADY was inferior to the $C_{18:1}$ fatty-acid supplemented ADY.

In all cases, reconstitution of ADYs at the lower temperature of 25°C (Suboptimal for the commercial product) resulted in diminished recovery of fermentative activity. ADYs that had been produced from yeasts grown in media supplemented with the $C_{18:1}$ fatty acid exhibited an increase in the percentage loss of fermentative activity within the range 8 -13%. Those that had been similarly grown in media supplemented with the $C_{18:2}$ fatty acid were more adversely affected by reconstitution at 25°C; the increased percentage loss of fermentative activity was 26.5%. It is important to note that the percentage dry matter of all the ADYs produced were similar, indicating that the drying process had produced a consistant and reproducible product.

EFFECT OF FATTY-ACID SUPPLEMENTATION OF *Saccharomyces cerevisiae* MATING STRAIN RP 108 ON THE LIPID COMPOSITION OF WHOLE CELLS

Supplementation of cultures of strain RP 108 with various fatty acids has been shown to alter the recovery of fermentative activity after drying. It was considered

essential to determine whether the type and concentration of fatty-acid supplementation employed had produced significant changes in the fatty-acyl composition of lipids extracted from whole cells. Furthermore,it was important to ascertain whether fatty-acid supplementation induced changes in the composition both of lipids that contained fatty-acyl residues (e.g. phospholipids) and other lipid components (e.g. free sterols).

Fatty-Acyl Composition

Table 7 shows the fatty-acyl composition of lipids extracted from whole cells of strain RP 108 grown in media supplemented with various fatty acids. Data obtained from analysis of lipid extracts of the cells of the Mating Parent strain HP 92 and Parent strain are provided for comparison. The concentration of $C_{18:1}$ Δ^9 cis included in cultures of strain RP 108 was reflected in the amount of the $C_{18:1}$ fatty-acyl residues found in the whole cell extracts. At the highest level of supplementation (1000mg/litre), $C_{18:1}$ fatty-acyl residues were the only unsaturated fatty-acyl residues detected in the cell and accounted for approximately 85% of the total fatty-acyl residues. This represented a gross distortion of the fatty-acyl composition when compared with the Mating Parent strain HP 92, where $C_{16:1}$ residues were the major fatty-acyl components. At the lowest level of $C_{18:1}$ fatty-acid supplementation (150mg/litre), the $C_{18:1}$ fatty-acyl content was diminished by approximately 75% and was similar to the value obtained for the Mating Parent strain HP 92.

TABLE 7

FATTY-ACYL COMPOSITION OF LIPIDS OF *Saccharomyces cerevisiae* MATING STRAIN RP 108

YEAST STRAIN	RP 108	RP 108	RP 108	RP 108	RP 108	HP 92 (MATING PARENT)	PARENT
FATTY-ACID SUPPLEMENT	$C_{18:1}$	$C_{18:1}$	$C_{18:1}$	$C_{18:1}$, $C_{16:0}$	$C_{18:2}$	NONE	NONE
mg/litre	1000	350	150	150 300	350	NONE	NONE
FATTY-ACYL RESIDUE $C_{14:0}$	t	t	t	t	t	t	t
$C_{15:0}$	t	t	t	t	t	t	t
$C_{16:0}$	0.549±0.06	1.21±0.26	1.02±0.19	2.93±0.48*	1.16±0.15	0.798±0.07	0.991±0.28
$C_{16:1}$	-	0.200±0.08	0.303±0.14	0.513±0.07	t	2.92±0.31	2.40±0.27
$C_{16:2}$	-	-	-	-	-	0.199±0.07	t
$C_{17:0}$	t	t	t	t	-	t	t
$C_{18:0}$	0.482±0.17	0.470±0.14	0.295±0.03	0.300±0.04	0.505±0.05	0.222±0.03	0.316±0.14
$C_{18:1}$	6.69±0.50*	4.45±0.41*	1.65±0.21*	1.60±0.15*	0.143±0.23	1.87±0.23	2.50±0.50
$C_{18:2}$	t	t	t	t	3.06±0.43*	t	-
$C_{18:3}$	-	-	-	-	t	-	-
TOTAL	7.77±0.42	6.40±0.81	3.29±0.46	5.39±0.63	5.04±0.54	6.04±0.55	6.39±1.06

Values given are the means of at least five replicates showing 95% confidence limits; mg/100mg dry wt. cells

t = Trace (< 1% of total) - = Not detected * Indicates fatty-acid supplement

Supplementation of cultures of RP 108 with 300mg $C_{16:0}$/litre (together with $C_{18:1}$ fatty acid) and with 350mg $C_{18:2}\ \Delta^{9,12}$ all <u>cis</u>/litre fatty acids, resulted in those residues becoming the major components detectable in the lipids of whole cells. Supplementation at high concentrations with either $C_{18:1}\ \Delta^{9}$ <u>cis</u> or $C_{18:2}\ \Delta^{9,12}$ all <u>cis</u> fatty acids appeared to suppress endogenous synthesis of $C_{16:1}$, and to a lesser extent, $C_{16:0}$ fatty-acyl residues. In contrast to these widely varying spectra of fatty-acyl compositions, the compositions of whole-cell extracts from the Mating Parent HP 92 and Parent strains were similar and both contained $C_{18:1}$ and $C_{16:1}$ residues as the major fatty-acyl residues.

It was clear that recovery of fermentative activity after drying could not simply be related directly to the proportion of $C_{18:1}$ residues or even to the proportion of unsaturated fatty-acyl residues present in whole cell extracts. Attempts were therefore made to determine the fate of the fatty-acid supplements by analysis of the phospholipid and neutral lipid fractions of whole cell lipid extracts (Table 8). The distribution of fatty-acyl supplements within the two classes of lipids varied considerably. The $C_{18:1}$ fatty-acid supplement was primarily required by the cell for phospholipid synthesis and was only found in the neutral lipids when supplied at the highest concentration (1000mg/litre). The percentage composition of the individual fatty-acyl residues, and the total percentage

TABLE 8

PERCENTAGE COMPOSITION OF THE FATTY-ACYL RESIDUES FROM THE PHOSPHOLIPIDS AND NEUTRAL LIPIDS OF *Saccharomyces cerevisiae* MATING STRAIN RP 108

YEAST STRAIN	RP 108		RP 108		RP 108	
FATTY-ACID SUPPLEMENT	$C_{18:1}$ 1000mg/litre		$C_{18:1}$ 350mg/litre		$C_{18:1}$ 150mg/litre	
LIPID FRACTION	NEUTRAL	PHOSPHOLIPID	NEUTRAL	PHOSPHOLIPID	NEUTRAL	PHOSPHOLIPID
FATTY-ACYL RESIDUE $C_{12:0}$	t	t	t	t	t	t
$C_{14:0}$	1.59±4.81	t	5.64±3.41	t	5.57±6.33	t
$C_{15:0}$	t	t	3.82±0.92	t	1.90±2.86	t
$C_{16:0}$	12.0±2.33	20.4±4.97	61.8±7.79	21.3±8.43	65.5±12.4	30.8±8.32
$C_{16:1}$	t	t	-	5.55±2.40	-	9.81±0.63
$C_{16:2}$	-	-	-	-	-	-
$C_{17:0}$	t	t	t	-	t	-
$C_{18:0}$	10.1±0.97	9.53±6.53	26.7±4.55	8.55±1.33	25.1±3.30	7.80±3.56
$C_{18:1}$	75.0±6.41*	69.1±8.43*	- *	62.3±5.46*	- *	51.3±4.28*
$C_{18:2}$	-	t	-	-	t	t
$C_{18:3}$	-	-	-	-	-	-
% UNSATURATED	75.0	69.1	0	67.9	0	61.1

Values given are the means of at least five replicates showing 95% confidence limits
t = Trace (< 1% of total) - = Not detected * Indicates fatty-acid supplement

TABLE 8 (continued)

YEAST STRAIN	RP 108		RP 108	
FATTY-ACID SUPPLEMENT	$C_{18:1}$ 150mg/litre $C_{16:0}$ 300mg/litre		$C_{18:2}$ 350mg/litre	
LIPID FRACTION	NEUTRAL	PHOSPHOLIPID	NEUTRAL	PHOSPHOLIPID
FATTY-ACYL RESIDUE $C_{12:0}$	t	t	t	t
$C_{14:0}$	2.15±7.33	t	1.39±0.24	t
$C_{15:0}$	1.36±0.82	t	t	t
$C_{16:0}$	88.6±10.4*	37.0±12.5*	18.2±1.97	31.8±3.98
$C_{16:1}$	-	15.3±3.41	2.14±0.82	1.32±0.94
$C_{16:2}$	-	-	-	-
$C_{17:0}$	1.37±1.43	-	-	-
$C_{18:0}$	5.36±7.45	7.23±1.75	9.49±0.92	11.8±20.4
$C_{18:1}$	- *	39.0±16.3*	4.54±2.29	2.13±0.72
$C_{18:2}$	-	t	62.1±5.84*	51.6±3.34*
$C_{18:3}$	-	-	1.27±0.28	t
% UNSATURATED	0	54.3	70.1	55.1

TABLE 8 (continued)

YEAST STRAIN	HP 92 (MATING PARENT)		PARENT	
FATTY-ACID SUPPLEMENT	NONE		NONE	
LIPID FRACTION	NEUTRAL	PHOSPHOLIPID	NEUTRAL	PHOSPHOLIPID
FATTY-ACYL RESIDUE $C_{12:0}$	t	t	1.36±0.39	t
$C_{14:0}$	4.15±1.81	1.10±0.09	8.81±1.69	t
$C_{15:0}$	2.04±1.43	1.10±1.00	3.81±0.68	t
$C_{16:0}$	38.3±5.19	25.6±7.93	56.0±6.20	16.4±3.54
$C_{16:1}$	19.3±2.59	34.9±6.82	1.79±3.83	35.9±4.43
$C_{16:2}$	1.22±1.34	2.85±0.58	-	t
$C_{17:0}$	t	t	1.12±2.64	-
$C_{18:0}$	13.9±4.55	9.13±3.27	25.6±3.40	4.90±1.30
$C_{18:1}$	19.5±4.77	25.5±4.32	t	40.7±3.06
$C_{18:2}$	t	t	-	-
$C_{18:3}$	-	-	-	-
% UNSATURATED	40.0	63.3	1.79	76.6

of unsaturated fatty-acyl residues of the phospholipids isolated from yeasts supplemented at the two high levels of supplementation (350 and 1000mg/litre) were similar. In contrast, the composition of the neutral lipids from these yeasts were dissimilar.

Supplementation of cultures of strain RP 108 with only 150mg $C_{18:1}$ Δ^9 cis/litre produced yeasts containing phospholipids that contained a lower percentage of unsaturated fatty-acyl residues, despite the appearance of greater proportions of $C_{16:1}$ residues which probably resulted from endogenous synthesis. Supplementation with 300mg $C_{16:0}$/litre, in addition to the $C_{18:1}$ acid, resulted in a further lowering of the proportion of unsaturated fatty-acyl residues in the phospholipids. However, the majority of the saturated supplement was found in the neutral lipids.

Analysis of fatty-acyl residues of the phospholipids and neutral lipids of yeasts grown in media supplemented with the $C_{18:2}$ acid revealed a different pattern of fatty-acid incorporation compared with yeasts grown in media supplemented with a similar concentration of the $C_{18:1}$ acid. Considerable proportions of $C_{18:2}$ ^{9,12} all cis were detected in both the phospholipids and neutral lipids. The proportion of unsaturated residues in the phospholipids of the $C_{18:2}$-supplemented cells was lower than that observed in the phospholipids of the $C_{18:1}$-supplemented cells.

Analysis of the phospholipids and neutral lipids in extracts of the Mating Parent strain HP 92 revealed a high proportion of unsaturated fatty-acyl residues (both $C_{18:1}$ and $C_{16:1}$) in both fractions. Results obtained from an analysis of the lipid fractions from the Parent strain were not similar to those obtained from strain HP 92. The neutral lipids contained a lower proportion of unsaturated fatty-acyl residues. However, the phospholipids contained the highest proportion of unsaturated fatty-acyl residues recorded during this study. The unsaturated residues were present as $C_{16:1}$ and $C_{18:1}$ in the proportions 0.9:1.0 approximately.

Other Lipid Components

Analyses of lipid components, other than fatty-acyl residues, was carried out on lipids from strain RP 108 grown in medium supplemented with various fatty acids. (Table 9) The total lipid content of all the yeasts were similar. Also, the total phospholipid contents of the mating strains HP 92 and RP 108 (with various fatty-acid supplements) were similar but lower than that in the extracts obtained from the Parent strain. The highest free sterol content in strain RP 108 was found in extracts from cells grown in medium supplemented with a low concentration (150mg/litre) of $C_{18:1}\ \Delta^{9}$ cis and 350mg $C_{18:2}\ \Delta^{9,12}$ all cis/litre. The lowest free sterol content was in extracts of cells from strain HP 92, which also possessed the lowest content of sterol ester. The sterol ester content appeared to increase with

TABLE 9

TOTAL LIPID, PHOSPHOLIPID, STEROL, STEROL ESTER AND FREE FATTY ACID COMPOSITION OF LIPIDS OF *Saccharomyces cerevisiae* MATING STRAIN RP 108 GROWN IN THE PRESENCE OF DIFFERENT FATTY ACIDS

YEAST STRAIN	RP 108	RP 108	RP 108	RP 108	RP 108	HP 92 (MATING PARENT)	PARENT
FATTY-ACID SUPPLEMENT	$C_{18:1}$	$C_{18:1}$	$C_{18:1}$	$C_{18:1}$, $C_{16:0}$	$C_{18:2}$	NONE	NONE
mg/litre	1000	350	150	150 300	350	NONE	NONE
TOTAL LIPID	11.7±1.34	12.0±2.25	11.1±1.28	11.7±1.74	11.3±2.54	11.4±1.92	11.6±1.00
TOTAL PHOSPHO-LIPID	3.14±0.28	3.08±0.31	3.08±0.22	3.07±0.26	3.07±0.13	3.59±0.26	4.12±0.30
FREE STEROL	0.194±0.19	0.145±0.02	0.201±0.05	0.173±0.14	0.288±0.08	0.075±0.02	0.357±0.18
STEROL ESTER	0.687±0.39	0.320±0.16	0.340±0.24	0.292±0.24	0.906±0.25	0.247±0.19	1.30±0.97
FREE FATTY ACID	0.159±0.06	0.211±0.08	0.177±0.05	0.435±0.19	0.145±0.08	0.159±0.05	0.147±0.04

Values given are the means of at least five replicates showing 95% confidence limits; mg/100mg dry wt. cells.

supplementation with increased concentrations of $C_{18:1}$ fatty acid. The Parent strain contained a considerably higher content of free sterol and sterol ester than either of the mating strains. Similar values for the free fatty acid content were obtained for strain HP 92, Parent strain and strain RP 108 grown in media supplemented with different concentrations of the $C_{18:1}$ and $C_{18:2}$ acids, indicating that little of the fatty acid supplements had remained 'free' and not incorporated into cell lipids.

The effect of fatty-acid supplementation of strain RP 108 on the contents of the individual phospholipids is shown in Table 10. Phosphatidylcholine (PC) and Phosphatidylinositol (PI) were the major phospholipids found in all strains. Cells of strain RP 108 grown in media supplemented with 1000 and 350mg of the $C_{18:1}$ acid/litre contained similar amounts of individual phospholipids as the Mating Parent HP 92 strain. Supplementation of media for strain RP 108 with lower concentrations of the $C_{18:1}$ acid (150mg/litre) produced cells with a diminished content of PC and PI, but with significantly increased contents of Phosphatidylserine (PS) and Phosphatidic acid (PA). Cells supplemented with the $C_{18:2}$ acid contained significant amounts of Cardiolipin (CL), together with a higher content of Phosphatidylethanolamine (PE) than cells grown in media supplemented with a smaller concentration of the $C_{18:1}$ fatty acid.

TABLE 10

PHOSPHOLIPID COMPOSITION OF CELL LIPID EXTRACTS OF Saccharomyces cerevisiae MATING STRAIN RP 108 GROWN IN MEDIA SUPPLEMENTED WITH DIFFERENT FATTY ACIDS

YEAST STRAIN	RP 108	RP 108	RP 108	RP 108	RP 108	HP 92 (MATING PARENT)	PARENT
FATTY-ACID SUPPLEMENT	$C_{18:1}$	$C_{18:1}$	$C_{18:1}$	$C_{18:1}$, $C_{16:0}$	$C_{18:2}$	NONE	NONE
mg/litre	1000	350	150	150, 300	350	NONE	NONE
LYSOPHOSPHO-LIPIDS	t	t	t	t	t	t	t
PHOSPHATIDYL-INOSITOL	1.13±0.14	1.11±0.18	0.915±0.21	0.985±0.09	0.841±0.23	1.28±0.16	1.03±0.17
PHOSPHATIDYL-SERINE	0.251±0.06	0.240±0.07	0.467±0.06	0.478±0.22	0.199±0.06	0.320±0.07	0.350±0.16
PHOSPHATIDIC ACID	t	t	0.271±0.13	0.164±0.05	0.046±0.01	t	t
PHOSPHATIDYL-CHOLINE	1.54±0.12	1.47±0.18	1.12±0.10	1.13±0.13	1.36±0.31	1.68±0.17	2.20±0.21
PHOSPHATIDYL-ETHANOLAMINE	0.262±0.05	0.227±0.07	0.217±0.06	0.199±0.07	0.428±0.16	0.271±0.09	0.526±0.06
CARDIOLIPIN	t	-	t	t	0.209±0.13	t	t

Values given are the means of at least five replicates showing 95% confidence limits; mg/100mg dry wt. cells. t = Trace (< 1% of total) - = Not detected

The composition of the free and esterified sterols present in whole cell lipid extracts (Table 11) indicated that the major sterols present were ergosterol and 24 (28)-dehydroergosterol. The latter sterol was the major component of the free sterols isolated from cells of strains HP 92 and RP 108 grown in media supplemented with each of the fatty acids excepting the $C_{18:2}$ acid. In contrast, ergosterol was the major sterol isolated from cells of the Parent strain.

PREPARATION AND ANALYSIS OF ISOLATED PLASMA MEMBRANES FROM *Saccharomyces cerevisiae* MATING STRAINS HP 92 AND RP 108 GROWN IN MEDIA SUPPLEMENTED WITH DIFFERENT CONCENTRATIONS OF $C_{18:1}\ \Delta^9$ cis AND $C_{18:2}\ \Delta^{9,12}$ all cis FATTY ACIDS

Preparation of Sphaeroplasts

Exo-β-(1-3)glucanase purified from Basidiomycete QM 806 was used routinely for preparation of sphaeroplasts from anaerobically grown cells of *Saccharomyces cerevisiae* NCYC 366 grown in media supplemented with different unsaturated fatty acids (Alterthum and Rose, 1973) and different sterols (Hossack and Rose, 1976). Although the nature of the fatty-acyl supplement employed was found to alter the susceptibility of the cell wall to digestion, Alterthum and Rose (1973) found that sphaeroplast formation was completed within 15-60min, using a reaction mixture with an enzyme/cell ratio of

TABLE 11

PERCENTAGE COMPOSITION OF THE FREE AND ESTERIFIED STEROLS OF *Saccharomyces cerevisiae* MATING STRAIN RP 108 GROWN IN MEDIA CONTAINING DIFFERENT FATTY ACIDS

YEAST STRAIN	RP 108	RP 108	RP 108	RP 108	RP 108	HP 92 (MATING PARENT)	PARENT
FATTY-ACID SUPPLEMENT	$C_{18:1}$	$C_{18:1}$	$C_{18:1}$	$C_{18:1}$, $C_{16:0}$	$C_{18:2}$	NONE	NONE
mg/litre	1000	350	150	150, 300	350	NONE	NONE
FREE STEROLS							
ZYMOSTEROL	19.6*	24.0*	8.47*	11.6	4.94	21.4	3.79*
ERGOSTEROL	32.2*	28.8*	35.9*	35.7	58.9	33.2	81.7*
24 (28)-DEHYDRO-ERGOSTEROL	47.9*	47.3*	59.1*	52.4	36.2	45.4	14.5*
ESTERIFIED STEROLS							
ZYMOSTEROL	10.5	15.9*	6.59	15.8*	3.82	24.4*	5.24
ERGOSTEROL	57.1	35.5*	35.4	33.4*	82.9	30.5*	61.3
24 (28)-DEHYDRO-ERGOSTEROL	33.7	48.6*	58.0	50.9*	13.4	45.2*	33.4

Values given are the mean of triplicate or duplicate* determinations.

5-10 units glucanase/mg dry wt. equiv. cells. Mating strain HP 92 was considerably more resistant to the action of glucanase than Sacch. cerevisiae NCYC 366. Complete sphaeroplast formation was only possible in reaction mixtures containing 20 units glucanase/mg dry wt.equiv. cells, and when the cells had been previously treated with mercaptoethanol (10mM; see Methods). Sphaeroplast formation was judged to be complete after 120-150 minutes (Figure 13). No increase in the rate of sphaeroplast formation was observed when higher enzyme/cell ratios were employed.

The times taken for sphaeroplast formation from cells of mating strains HP 92 and RP 108 (grown in media supplemented with different fatty acids) varied considerably, but were not consistant. Values observed were within the range 90-170 minutes.

Preparation and Analysis of Isolated Plasma Membranes

Plasma membranes from sphaeroplasts of Sacch. cerevisiae mating strains HP 92 and RP 108 grown in media supplemented with $C_{18:1}\,\Delta^{9}$ cis (1000,350 and 150mg/litre) and $C_{18:2}\,\Delta^{9,12}$ all cis (350mg/litre) fatty acids, were radio-actively labelled with ^{125}I, washed and separated on a discontinuous sucrose density gradient.

The proportion of ^{125}I that became bound to the plasma membranes of different batches of sphaeroplasts was found to vary considerably (Table 12). However, when

FIGURE 13

TIME COURSE OF SPHAEROPLAST FORMATION OF Saccharomyces cerevisiae MATING STRAIN HP 92

Sphaeroplast formation was monitored by diluting portions (0.1ml) of the cell suspension in glucanase-containing buffered sorbitol (1.4M) into 2.9ml water, gently shaking the diluted suspension and measuring the absorbance (E^{600}_{1cm}) after 10min. Control dilutions were made into 2.9ml buffered sorbitol.

■——■ 20 units glucanase/mg dry wt. equiv. cells

▲——▲ As above; cells previously treated for 15 min at 30°C in buffered sorbitol (1.4M) containing mercaptoethanol (10mM)

●——● Control; Portions (0.1ml) of cells in glucanase-containing buffered sorbitol diluted in 2.9ml buffered sorbitol (1.4M)

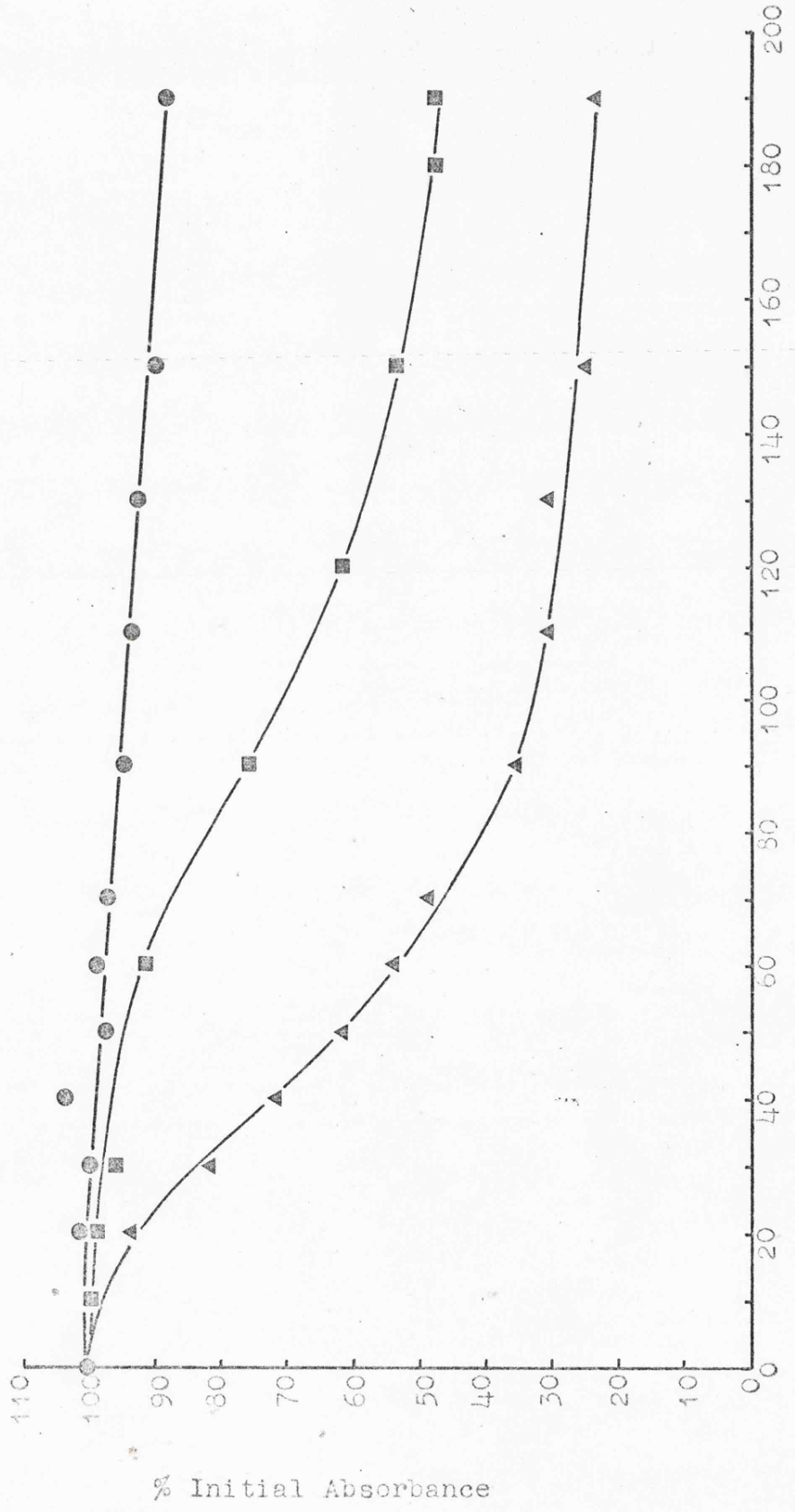

% Initial Absorbance
110
100
90
80
70
60
50
40
30
20
10
0
20
40
60
80
100
120
140
160
180
200
Incubation time (min)

TABLE 12

DISTRIBUTION OF RADIOACTIVITY AND PROTEIN ASSOCIATED WITH INTACT SPHAEROPLASTS AND PLASMA MEMBRANE FRACTIONS ISOLATED FROM *Saccharomyces cerevisiae* MATING STRAINS HP 92 AND RP 108 GROWN IN THE PRESENCE OF DIFFERENT FATTY ACIDS

YEAST STRAIN	RP 108	RP 108	RP 108	RP 108	HP 92 (MATING PARENT)
FATTY-ACID SUPPLEMENT	$C_{18:1}$	$C_{18:1}$	$C_{18:1}$	$C_{18:2}$	NONE
mg/litre	1000	350	150	350	NONE
Percentage of radioactivity associated with washed sphaeroplasts after labelling with ^{125}I.	8.70	4.98	6.60	31.7	14.3
Percentage of total radioactivity applied to gradient associated with the membrane pellet.	60.4	56.5	54.6	85.0	80.0
Percentage of total protein applied to the gradient associated with the membrane pellet.	16.8	24.5	23.5	11.9	12.9

Values given are the means of duplicate determinations.

the sphaeroplast lysates were applied to the gradient as described in Methods, the highest concentration of radioactivity was found in the pellet at the bottom of the gradient with a density just greater than 1.30g/cm^3. The pellet also contained the highest concentration of protein observed in the fractions (Figure 14) and accounted for some 11-25% of the total protein applied to the gradient (Table 12). Figure 14 is presented as a typical set of results obtained during the separation of plasma membranes from sphaeroplast lysates.

After sucrose density-gradient centrifugation the pellets were freeze-dried and the percentage composition of the fatty-acyl residues determined (Table 13). The percentage composition of fatty-acyl residues isolated from extracts of whole cell lipids and phospholipids are provided for comparison (Data adapted from Tables 7 and 8). In general, analysis of the fatty-acyl residues from the phospholipids closely resembled the fatty-acyl composition of the isolated plasma membranes. A similar relationship between total cell lipid and plasma membrane fatty-acyl compositions existed for cells that had been grown under conditions of submaximal fatty-acid supplementation ($C_{18:1}$, 150mg/litre; $C_{18:1}$, 350mg/litre; $C_{18:2}$, 350mg/litre) However, under conditions of maximal fatty-acid supplementation ($C_{18:1}$, 1000mg/litre), the fatty-acyl composition of the total cell lipid extract differed from the composition of the plasma membrane preparation. A similar relationship between the fatty-

FIGURE 14

HISTOGRAM SHOWING DISTRIBUTION OF RADIO-ACTIVITY AND PROTEIN ON A SUCROSE DENSITY GRADIENT OF A LYSATE OF RADIO-ACTIVELY LABELLED SPHAEROPLASTS OF Saccharomyces cerevisiae MATING STRAIN RP 108 GROWN IN MEDIA SUPPLEMENTED WITH $C_{18:2}\ \Delta^{9,12}$ all cis (350mg/litre).

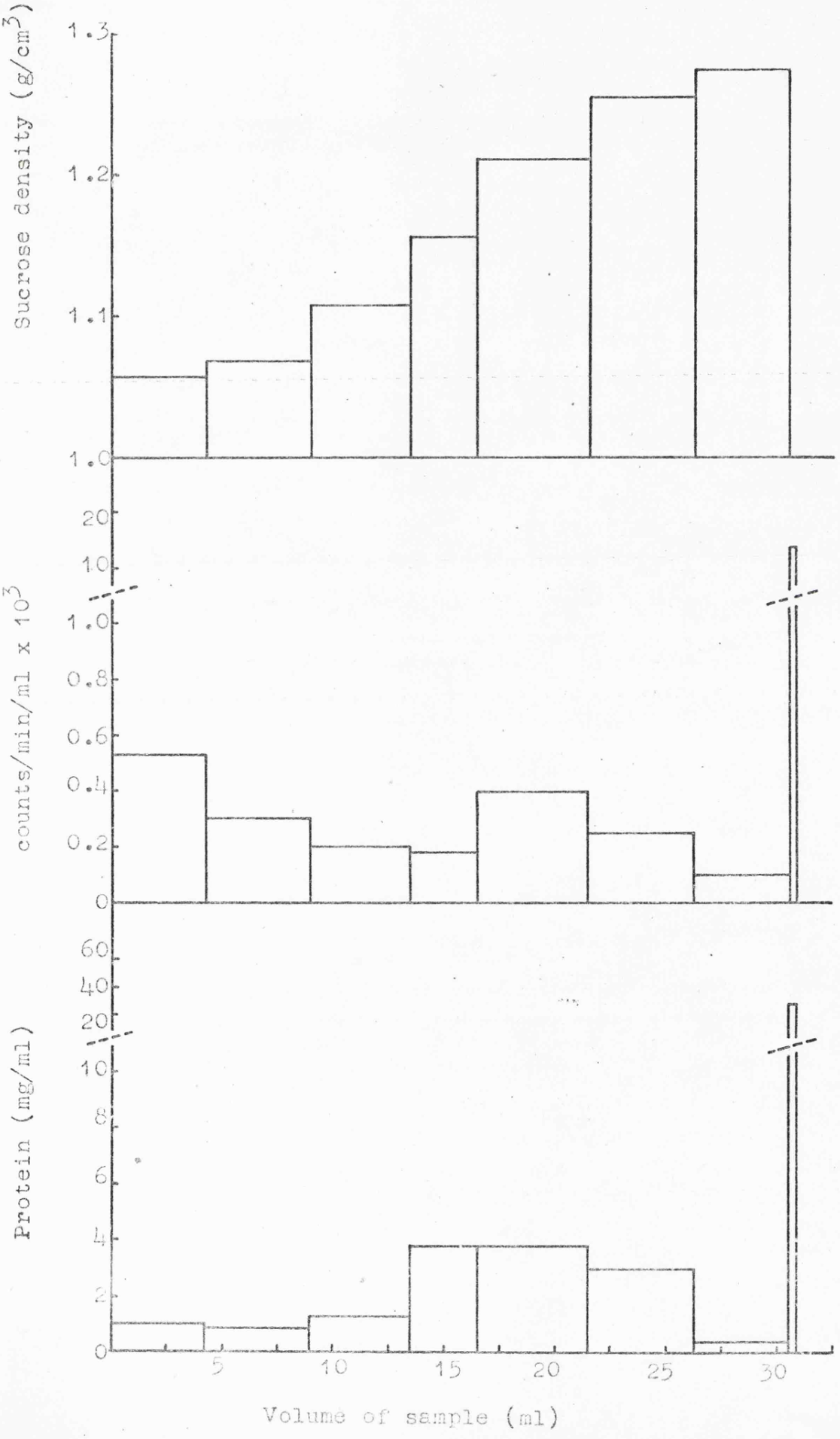

Sucrose density (g/cm3)
1.3
1.2
1.1
1.0
20
10
counts/min/ml x 10^3
1.0
0.8
0.6
0.4
0.2
0
60
40
20
Protein (mg/ml)
10
8
6
4
2
0
5
10
15
20
25
30
Volume of sample (ml)

TABLE 13

PERCENTAGE COMPOSITION OF FATTY-ACYL RESIDUES FROM TOTAL CELL LIPIDS, PHOSPHOLIPIDS AND PLASMA MEMBRANES OF Saccharomyces cerevisiae MATING STRAIN RP 108 GROWN IN THE PRESENCE OF DIFFERENT FATTY ACIDS

TC = Total cell lipid extract

PL = Phospholipid fraction

M = Plasma membrane preparation

(a - Mean values determined from at least five replicates

b - Mean values determined from duplicate preparations)

YEAST STRAIN	RP 108			RP 108			RP 108			RP 108			HP 92 (MATING PARENT)		
FATTY-ACID SUPPLEMENT	$C_{18:1}$			$C_{18:1}$			$C_{18:1}$			$C_{18:2}$			NONE		
mg/litre	1000			350			150			350			NONE		
LIPID EXTRACT	TC	PL	M	TC	PL	M	TC	PL	M	TC	PL	M	TC	PL	M
FATTY-ACYL RESIDUE $C_{12:0}$	t	t	t	t	t	t	t	t	2.1	t	t	1.6	t	t	2.9
$C_{13:0}$	-	-	t	-	-	t	-	-	t	-	-	1.6	-	-	1.3
$C_{14:0}$	t	t	2.4	t	t	1.1	t	t	1.9	t	t	3.3	t	1.1	4.1
$C_{15:0}$	t	t	t	t	t	t	t	t	t	t	t	1.6	t	1.1	1.6
$C_{16:0}$	7.1	20.4	15.3	18.9	21.3	19.2	31.0	30.8	26.1	23.0	31.8	25.4	13.2	25.6	22.8
$C_{16:1}$	-	t	t	3.1	5.6	1.1	9.2	9.8	11.8	t	1.3	3.4	48.3	34.9	20.8
$C_{16:2}$	-	-	-	-	-	-	-	-	-	-	-	-	3.3	2.9	6.0
$C_{17:0}$	t	t	-	t	-	-	-	-	-	-	-	-	-	t	-
$C_{18:0}$	6.2	9.5	7.2	7.3	8.6	5.0	9.0	7.8	5.6	10.0	11.8	7.5	3.7	9.1	6.0
$C_{18:1}$	86.1	69.1	71.8	69.5	62.3	65.9	50.2	51.3	40.8	2.8	2.1	14.8	31.0	25.5	27.1
$C_{18:2}$	-	t	2.0	-	-	5.6	-	t	9.7	60.7	51.6	40.8	-	t	13.5
% UNSATURATED	86.1	69.1	73.8	72.6	67.9	72.6	59.4	61.1	62.3	63.5	55.1	59.0	82.6	63.3	61.4
	a	a	b	a	a	b	a	a	b	a	a	b	a	a	b

acyl compositions of the lipid fractions and plasma membranes can be seen for Mating Parent strain HP 92.

Although $C_{18:2}$ residues were either not detected, or found only in trace amounts in total cell and phospholipid fractions, they were found in appreciable proportions in isolated plasma membranes.

RECONSTITUTION IN THE PRESENCE OF SPERMINE OF ACTIVE DRIED YEASTS PREPARED FROM *Saccharomyces cerevisiae* MATING STRAINS HP 92 AND RP 108 GROWN IN MEDIA SUPPLEMENTED WITH VARIOUS CONCENTRATIONS OF UNSATURATED FATTY ACIDS

Spermine, a polyamine, has been used to stabilise yeast and bacterial osmotically-sensitive cells, protoplasts and subcellular components (Bachrach, 1973; Alterthum and Rose, 1973). The solubilising action of spermine is probably due to its ability to bind with negatively charged membrane components and in so doing, to act as a'brace' on the membrane at the molecular level.

The effect of reconstitution at 38°C in spermine (10mM) of ADYs produced from cells of mating strains HP 92 and RP 108 grown in media supplemented with different fatty acids is shown in Figure 15. Reconstitution of ADYs in the presence of spermine resulted in a marked diminution in the recovery of fermentative activity. The greatest

FIGURE 15

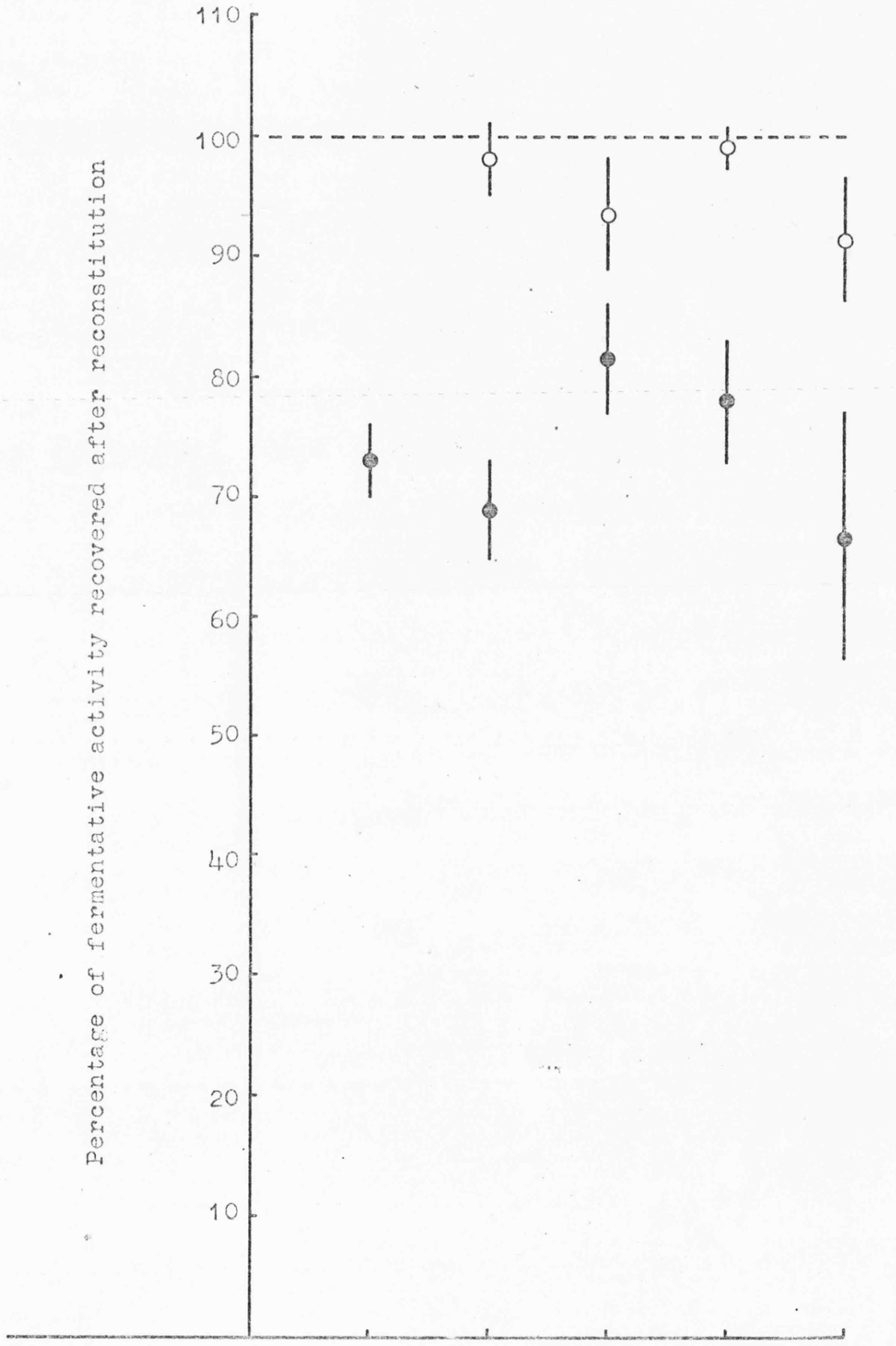
EFFECT OF SPERMINE ON RECONSTITUTION OF ACTIVE DRIED YEAST PRODUCED FROM *Saccharomyces cerevisiae* MATING STRAINS HP 92 AND RP 108 GROWN IN MEDIA SUPPLEMENTED WITH DIFFERENT UNSATURATED FATTY ACIDS

Values obtained by reconstitution at 38°C for 15min in distilled water were taken as 100%.

Values quoted are the means of between three and six replicates showing 95% confidence limits:

● = Reconstitution in spermine (10mM).

○ = Reconstitution in water but the equivalent quantity of spermine added to the reaction mixture in the Warburg flasks during determination of fermentative activity.


Percentage of fermentative activity recovered after reconstitution
110
100
90
80
70
60
50
40
30
20
10
Yeast strain
HP 92
RP 108
RP 108
RP 108
RP 108
Fatty acid supplement
NONE
$C_{18:1}$
$C_{18:1}$
$C_{18:1}$
$C_{18:2}$
mg/litre
NONE
1000
350
150
350

effect was observed with ADYs that contained a high proportion of unsaturated fatty-acyl residues in their total cell lipid extracts, (Table 13). The presence of spermine in the medium employed for the measurement of fermentative activity had only a marginal deleterious effect on the fermentative enzyme systems of reconstituted yeast cells.

DISCUSSION

Some of the data reported in this thesis were obtained by exploiting the requirement of a mutant of Saccharomyces cerevisiae for an unsaturated fatty acid when grown aerobically. Changes in cell lipid composition of yeast may also be induced by exploiting the anaerobic requirment of yeast for sterol and an unsaturated fatty acid (Andreason and Stier, 1954; Alterthum and Rose, 1973; Hossack and Rose, 1975). However, considerable care should be taken when comparing data obtained from yeasts grown under anaerobic conditions with aerobically-grown yeasts due to the considerable morphological and physiological differences between the two types of cell. Indeed, "the inability of glucose-repressed anaerobic cells of Sacch. cerevisiae to form mono-unsaturated acids would seem to be a 'retrograde step' and appears to result in a primative type of cell with lipid properties and membranes system intermediate between those of bacteria (prokaryates) and eukaryotic cells of animals and plants" (Rattray et al., 1975).

The approach taken during this study has been to obtain from a commercial yeast strain used for ADY production, (a) a mating parent strain, (b) derive from the mating strain an unsaturated fatty-acid auxotrophic strain namely RP 108 and, (c) utilise the properties of these strains to investigate the effect of altered fatty-acyl compositions of cell lipids and plasma membranes on the response of cells to the stress of drying and reconstitution.

This approach applied to a study of ADY is to my knowledge unique. However, the production and characterisation of desaturase mutants of fungi (both Saccharomyces sp. and Neurospora sp.) together with studies of lipid-requiring Mycoplasma sp. have been a field of active research for several years. Data obtained from the series of yeast desaturase mutants including Sacch. cerevisiae strains KD 115 and KD 40 isolated by Resnick and Mortimer (1966) and later exploited by Keith and his coworkers (Keith et al., 1973) are probably most relevant to a discussion of the characteristics of Sacch. cerevisiae strain RP 108.

Characteristics of Saccharomyces cerevisiae strain RP 108.

Saccharomyces cerevisiae mating strains HP 92 and RP 108 both exhibited characteristics of haploid strains (i.e. they were non sporulating strains that exhibited a specific mating type when cells were mixed with known haploid strains. In addition they gave lower cell yields than the Parent strain from which they were derived, however, it is conceivable that despite this circumstantial evidence, both these strains could contain more than one copy of some genetic information in the genome if the original commercial yeast strain was polyploid. Polyploidy and aneuploidy are frequently encountered in brewer's strains of Sacch. cerevisiae. The supposition that the two mating strains are true haploids could only be confirmed or otherwise by further genetic analysis.

Both the Parent and Mating Parent strain HP 92 were shown

to be respiratory competant using the method of Nagai (1963). Strain RP 108 was also found to be respiratory competant when grown in media supplemented with unsaturated fatty acid ($C_{18:1}\,\Delta^9$ cis) but after prolonged incubation on fatty-acid free medium exhibited characteristics of a respiratory incompetant strain, due probably to lowered mitochondrial efficiency under conditions of unsaturated fatty-acid depletion. A supply of unsaturated fatty acid (as Tween 80) to Sacch. cerevisiae KD 115 has been found to be essential for, (a) the development of mitochondrial respiratory function as shown by the development of cytochromes a a_3, b and c + c_1 (Gordon et al., 1972) and the maintenance of mitochondrial oxidative phosphorylation coupled to respiration (Proudlock et al., 1969; Haslam et al., 1973) and (b) suppression of respiratory incompetant (petite) mutants when grown in glucose-limited continuous culture (Marzuki et al., 1974). Attempts to distinguish respiratory incompetance by the inability to use a non-fermentable source of carbohydrate were inconclusive as both the Parent and mating strains exhibited poor growth on YEPGlycerol medium.

It is interesting to note that, while no specific attempts were made during this study to isolate a revertant of strain RP 108, routine checking of cultures did not reveal the presence of revertants. Marzuki et al. (1974) have stated that only cultures of strain KD 115 containing $< 0.1\%$ revertants were used in their studies, thus suggesting that strain KD 115 may be considerably less

genetically stable than strain RP 108.

Growth of strain RP 108 has been shown to be prompted by supplementation of the growth medium with oleic acid ($C_{18:1}$ Δ^9 cis). The requirement for such a fatty-acid supplementation was not absolute for cell growth. Indeed, cells of this strain grown in unsupplemented medium contained small but significant quantities of $C_{18:1}$ and $C_{16:1}$ fatty-acyl residues. This observation indicates that strain RP 108 possesses an operative desaturase system which, for reasons at present unknown, is unable to function sufficiently to satisfy the requirements of the cell. The desaturase system in Saccharomyces sp. is located in the intracellular microsomal fraction and organised into particles (Bloomfield and Bloch, 1960), and has been termed desaturase system I to distinguish it from system II employed by plants for desaturation of acyl-carrier protein complex. Desaturase system I has been identified in mammalian cells (rat and hen liver) and has been fractionated into several subparticles (Holloway and Wakil, 1970). These workers have reported that desaturase system I requires three enzymes for conversion of stearoyl - CoA to oleic acid (CoA): a desaturase specific for stearoyl - CoA, an NADH cytochrome b_5 reductase and cytochrome b_5, together with molecular oxygen. One may postulate that the observed deficiency in the desaturase system of Sacch. cerevisiae strain RP 108 is associated with a structural change in either the desaturase enzyme itself or the NADH cytochrome b_5

reductase, or a diminished availability of either of these enzymes and/or cytochrome $\underline{b}_5$.

The similarity of the genetic loci or desaturase deficiency of strains RP 108 and KD 115 could be compared in recombination experiments using a substantial stock of desaturase mutants (such as the KD series) as described by Keith et al. (1969). Despite practical difficulties concerning the availability to cells in culture of saturated fatty acids ($C_{16:0}$, $C_{18:0}$) suspended in aqueous growth medium, the data reported here do not support the possibility that strain RP 108 is a chain-elongation mutant, with a deficient fatty-acid synthetase system, similar to yeast strains reported by Henry and Keith (1971).

The data reported in this thesis can only serve as a preliminary indication of the specific fatty-acid requirement of strain RP 108 and more work would be required for firm conclusions to be drawn. However, the results indicate that the unsaturated fatty-acid requirement of this strain may be satisfied by other mono-unsaturated long-chain fatty acids, of varying chain length, position and number of double bonds, and structural configuration of the double bond (i.e. cis or trans). Cells routinely subcultured on $C_{18:1}$ Δ^9 cis fatty-acid supplemented medium required a period of metabolic re-adjustment (observed as a low growth rate) before they were able to utilise the different unsaturated fatty acid supplied and

give a relatively high cell yield. In the extreme case when cells were first supplemented with $C_{16:1}\,\Delta^{9}$ cis or $C_{18:2}\,\Delta^{9,12}$ all cis, growth was inhibited. Presumably the presence of high concentrations of these fatty acids suppresses on essential metabolic pathway (possibly long-chain fatty-acid synthesis) although why this phenomenon was not observed in $C_{18:1}\Delta^{9}$ cis - supplemented cultures is difficult to explain.

Comparing my results with those reported for Sacch. cerevisiae KD 115 (Wisnieski et al., 1970; Keith et al., 1973) is difficult as I am of the opinion that their results are open to more than one interpretation. Firstly, KD 115 was reported to have an absolute requirement for an unsaturated fatty acid and was not considered to be 'leaky' (i.e. little or no growth was observed in un-supplemented cultures; Wisnieski et al., 1970). However, these workers state that all cultures contained a detergent (Tergitol NP 40; 1%) to solubilise fatty-acid supplements, and they confirmed this detergent was not inhibitory to the growth of wild-type haploid cells (strain S288C). My evidence, with strain RP 108, is that cells deprived of unsaturated fatty acid (i.e. from unsupplemented cultures) are highly susceptible to the action of detergents due presumably to an altered fatty-acyl content in the lipids of the plasma membrane, and would not grow under such conditions. Employing media containing a detergent would provide results that would indicate that strain RP 108 had an absolute requirement

for an unsaturated fatty acid.

Secondly, Wisnieski *et al.* (1970) concluded from their results that strain KD 115 required supplementation with a fatty acid possessing unsaturation of the Δ^9 *cis* conformation only, although fatty acids containing unsaturations at different positions in the acyl chain, and of the *trans* configuration, were tested. These results were modified when the experiments were repeated (Keith *et al.*, 1973) to include fatty acids with unsaturations, at different positions, of different numbers and in the *trans* configuration (see Introduction). They explained these discrepancies as being due to an 'initial permeability barrier or some other metabolic restriction that it (KD 115) later lost' or to undetected impurities in the fatty acids used in the first experiments. Such revelations are disconcerting. I consider that it is conceivable that strain KD 115 could become adapted to accept other unsaturated fatty acids (as discussed previously) or that a significant genetic change has occurred in stocks of the strain during the two sets of experiments; based on the apparently high rate of genetic instability reported by Marzuki *et al.* (1974).

Data reported in this thesis suggest that polyunsaturated fatty acids are susceptible to auto-oxidation and hydroperoxide formation in aerobic yeast cultures. By-products from such reactions are toxic to yeast cell growth (see Results). This phenomenon has not been considered by Keith *et al.*(1973) even though they have been experimenting

with unsaturated fatty acids containing up to four double bonds. Susceptibility to hydroperoxide formation in unsaturated fatty acids is known to increase rapidly with an increase in the number of double bonds in the acyl chain (Lundberg and Jarvi, 1968) and could significantly influence the results obtained in cultural studies employing such fatty acids.

The effect of supplementation with fatty acids of wild-type (non lipid requiring) yeasts has been reported (Suomalainen and Keranen, 1968; Keith et al., 1969). These data mainly concerned supplementation with unsaturated fatty acids ($C_{16:1}$ and $C_{18:1}$). Suomalainen and Keranen (1968) reported increased enrichment of cell-lipid fatty acids of some 9 - 30% when a nutrient enriched medium was used. Keith et al. (1969), however, reported much higher levels of fatty-acid enrichment (40 - 65% approx.) when a minimal growth medium was employed. These results illustrate the fact that yeasts that do not possess a requirement for a fatty-acid may nevertheless absorb such fatty acids from the growth medium. One observation by Suomalainen and Keranen (1968) indicated that a saturated fatty acid ($C_{18:0}$) could be taken up by yeast cells. With the exception of this report there appears to be no evidence for the uptake of saturated fatty acids (particularly by the KD series of mutants). Data reported in this thesis shows that a yeast strain (RP 108) may absorb a saturated fatty acid ($C_{16:0}$) for which it has no obligate requirement.

This phenomenon has proved a useful additional method by which yeast fatty-acyl composition may be altered, however, the significance of these data are discussed more fully further on.

Lipid Composition of Saccharomyces cerevisiae Enriched with Various Fatty Acids

Cells of mating strains were grown under identical conditions (temperature, aeration, mixing, nutrient supply and as far as can be ascertained pH value). Growth was limited by the supply of glucose and other nutrients and not by the availability of unsaturated fatty acids which were supplied in excess. Cells of the Parent strain were grown under similar conditions except that the period of growth was 24h compared with 36h for the mating strains. Apart from growing at gradually diminishing growth rate, cells were also subject to periods of nitrogen and probably phosphorus limitation. Nevertheless, the growth method produced yeasts of consistant characteristics (cell yield, nitrogen and phosphorus contents and fermentative activity). The effects of different growth conditions on the lipid composition of yeasts has been briefly reviewed in the Introduction.

It would appear that the fatty-acyl residues from the acids were incorporated into yeast cells in chemically

unmodified forms, as analysis of fatty-acyl residues from total cell lipids reflected the type and concentrations of fatty-acid supplement employed. These data confirm the conclusions of other workers (e.g. Keith et al., 1973) that yeastsdo not possess an oxidative pathway for metabolising fatty acids as an energy source. The degree of incorporation of fatty-acid supplements was high (approx. 70%) although it might prove possible to improve this value by careful choice of a solubilising agent in the growth medium, thus preventing deposition of hydrophobic fatty acid on the surfaces of the culture vessel.

Extensive modification of the fatty-acyl composition of total cell lipids was obtained by supplementation of the medium with various concentrations of $C_{18:1}\Delta^{9}$ cis and $C_{18:2}\Delta^{9,12}$ all cis fatty acids. Under conditions of maximum supplementation, the $C_{18:1}$ fatty-acyl content of mutant cells was increased 3.7-fold when compared with the Mating Parent strain HP 92, and was the only unsaturated fatty-acyl residue present, accounting for some 86% of the total fatty-acyl residues in extracts. This degree of supplementation was greater than that observed by Alterthum and Rose (1973; 65% $C_{18:1}$ fatty-acyl residues in the total cell lipids) and by Hossack (1975; 58-64% $C_{18:1}$ fatty-acyl residues in the phospholipids), both workers having utilised the requirement of anaerobically grown yeast for an unsaturated fatty acid and a sterol in

the growth medium.

Endogenous synthesis of $C_{16:1}$ residues was suppressed by supplementation with high concentrations (1000 and 350mg/litre) $C_{18:1}\Delta^{9}$ cis and $C_{18:2}\Delta^{9,12}$ all cis fatty acids, although small but significant quantities of $C_{16:1}$ residues were present in yeasts supplemented with less $C_{18:1}\Delta^{9}$ cis acid. From this observation one may conclude that the desaturation of $C_{16:0}$ to $C_{16:1}$ and $C_{18:0}$ to $C_{18:1}$ is probably effected by the same desaturase system controlled by one regulatory system. In wild-type yeasts, the proportion of $C_{16:1}/C_{18:1}$ fatty-acyl residues is probably controlled by the availability of the appropriate saturated fatty acid. Indeed, the inital step of de novo fatty acid biosynthesis involving acetyl-CoA carboxylase has been suggested as being under negative feedback control by long-chain fatty-acyl CoA (Sumper, 1974). This hypothesis, although logical, may prove to be an over simplification of the regulatory mechanism involved.

The percentage of unsaturated fatty-acyl residues in the whole cell lipid extracts of the Mating Parent strain (HP 92; 82.6%) and the mating strain RP 108 grown in media supplemented with 1,000mg $C_{18:1}\Delta^{9}$ cis acid/litre (86.1%) are similar but slightly higher than the value (77.0%) reported for Sacch. cerevisiae NCYC 366 grown slowly ($0.05h^{-1}$) at 30°C (Hunter and Rose, 1972).

Analysis of the fatty-acyl residues from the phospholipids

and neutral (or non-polar) lipids from cells of strain RP 108 supplemented with various fatty acids has yielded interesting information concerning the fate of the fatty-acid supplement within the cell. Cells from cultures supplemented with $C_{18:1}\ \Delta^9$ *cis* fatty acid $\leqslant$ 4.45mg/100mg dry cells, incorporate all the supplement into the phospholipids. Similarly, any endogenously produced $C_{16:1}$ fatty-acyl residues present in the cells were found in the phospholipids. Under such conditions, unsaturated fatty-acyl residues were not found in the neutral lipids. Under conditions of high fatty-acid supplementation of the media (cells contained approx. 7.0 mg $C_{18:1}\ \Delta^9$ *cis* fatty acid /100mg dry wt.) a high proportion of the fatty acid supplied was found in the neutral lipids. It would appear that unsaturated fatty acids are primarily required by the cell for phospholipid, and hence membrane, synthesis. Only when excess unsaturated fatty acid is available is it incorporated into neutral lipids, (sterol ester and triacylglycerols), which are thought to be located in low-density vesicles (Hossack *et al.*, 1973; Clausen *et al.*, 1974) or associated with a lipid carrier molecule (Sumper and Trauble, 1973) to facilitate the transport of fatty-acyl residues from the microsomal site of synthesis to the growing membrane. It has been proposed that saturated fatty-acyl chains are synthesised and incorporated into phospholipids within the membranes prior to desaturation (Pugh and Kates, 1973; Talamo *et al.*, 1973), in which case unsaturated fatty acids supplied to a desaturase mutant yeast would be converted to the coenzyme A ester and incorporated directly into the membrane phospholipids.

The high content of unsaturated fatty-acyl residues achieved in lipids of strain RP 108 were similar to those reported by Trevelyan (1966) who studied the lipid composition of baker's yeast grown in several different media, one of which produced yeast of low nitrogen content. However, direct comparison of the percentage of unsaturated and saturated fatty-acyl residues from the phospholipids and neutral lipids of the Parent strain of this study and low-nitrogen yeast of Trevelyan (1966) revealed some considerable differences. The total percentage of unsaturated fatty-acyl residues from the phospholipids of these strains was similar (76 and 82% respectively), whereas the unsaturated fatty-acyl contents from the neutral lipids were approx. 2 and 83% respectively. It should be stressed, however, that the methods used by Trevelyan (1966) for lipid extraction and analyses were different to those employed in this study.

The total phospholipid contents of cells of strain RP 108 grown in media containing various concentrations of fatty acid were similar, but slightly lower, than those of the Mating Parent strain HP 92 and approximately 25% less than that observed in the Parent strain. The value obtained for the last strain agreed with that published for Sacch. cerevisiae NCYC 366 grown slowly ($0.05h^{-1}$) at $30^{o}C$; (4.08mg/100mg dry wt. cells; Hunter and Rose, 1972) and with that published for low-nitrogen yeast (51 μM phosphorus/g yeast ≡ 4mg phospholipid/100mg yeast; Trevelyan, 1966).

The composition of the phospholipids of strain RP 108 was found to vary with fatty-acid supplement. In all yeast-crops examined in this study, the two major phospholipids were phosphatidylcholine and phosphatidyl-inositol together with lesser quantities of phosphatidyl-serine, phosphatidylethanolamine, phosphatidic acid and cardiolipin. These analyses substantially agree with those data published for aerobically grown Sacch. cerevisiae NCYC 366 (Hunter and Rose, 1972) and for low-nitrogen Sacch. cerevisiae (Trevelyan, 1966).

Diminished supplementation of media for the mating strain RP 108 with unsaturated fatty acid ($C_{18:1}\Delta^{9}$ cis, $C_{18:2}\Delta^{9,12}$ all cis) resulted in a slight lowering of the contents of the two major phospholipids (PC and PI), together with a significant increase in the contents of phosphatidylserine (40-50%) and phosphatidic acid. Cardiolipin (diphosphatidylglycerol) was only detected in significant proportions in lipid extracts from cells grown in media supplemented with $C_{18:2}\Delta^{9,12}$ all cis fatty acid. Phosphatidic acid is one of the major intermediates in the synthesis of the major phospholipids (PC, PI, PS, PE; Rattray et al., 1975) from sn-glycero-3-phosphoric acid. The occurrence of phosphatidic acid in relatively large quantities suggested that the various molecular species of PA (i.e. the types of fatty-acyl chains included in the molecule) were unsuitable for further convertion to phospholipids. One may postulate that the PA isolated from cells grown with a restricted

quantity of $C_{18:1}\Delta^9$ cis fatty acid contained mainly saturated residues synthesised endogenously. Such a postulate would be interesting to examine.

The contents of free fatty acids isolated from cells grown in media supplemented with unsaturated fatty acid and cells of unsupplemented parent strains were low (cf. Hunter and Rose, 1972), indicating that the observed accumulation of unsaturated fatty-acyl residues by supplemented cells was by incorporation of these residues into cell lipids rather than by accumulation of intracellular 'pools' of free fatty acid. Only cells of strain RP 108 grown in media supplemented with $C_{16:0}$ accumulated relatively high quantities of free fatty acid, which perhaps reflects the poor incorporation of that fatty acid into cell lipids.

Some variation was observed in the contents of free sterols in the lipid extracts of cells of strain RP 108 grown in media supplemented with various fatty acids; however, the sterol content did not appear to be consistently altered by an increase in the content of $C_{18:1}\ \Delta^9$ cis fatty-acyl residues in the cell lipids and membranes. Cells grown in media supplemented with $C_{18:2}\Delta^{9,12}$ all cis fatty acid contained considerably more free sterol than cells grown in media supplemented with a similar quantity of $C_{18:1}\Delta^9$ cis acid. This observation may be a response of the cell to the presence of highly unsaturated fatty-acyl residues in the cell

membranes (which would consequently be more 'fluid' due to the perturbations produced in the packing of phospholipid acyl-chains by polyunsaturated fatty-acyl chains). Such increased fluidity may induce increased sterol synthesis and deposition within the cell membranes in an attempt by the cell to maintain a stable membrane structure with optimum physiological characterisitics required for essential membrane functions, similar to that proposed by Proudlock et al. (1968). Since cells of the Mating Parent strain HP 92 contained the least amount of free sterol, one may conclude that the membranes of these yeasts required little stabilising by free sterols. However, the results obtained contrast markedly with those values obtained from the Parent strain which contained some four to five times as much free sterol. Unfortunately, the contents of free sterols in cells of Sacch. cerevisiae grown anaerobically and supplemented with ergosterol and various unsaturated fatty acids have not been published.

The contents of esterified sterols increased approximately two-fold when cells of strain RP 108 were grown in media supplemented with excess $C_{18:1}\Delta^{9}$ cis fatty acid. Even greater quantities of esterified sterol were obtained from cells grown in media supplemented with $C_{18:2}\Delta^{9,12}$ all cis fatty acid. These data probably indicate the accumulation of intracellular 'pools' of stored fatty-acyl residues bound to sterol molecules, which were excess to the requirements of the cells for phospholipid and membrane synthesis.

Analysis of the individual sterols found as free or esterified sterols was not as extensive as analyses of other lipid classes. However, in all yeasts examined in this study, the major sterols were ergosterol and 24 (28)-dehydroergosterol, together with lesser quantities of zymosterol which is an intermediate in the synthesis of ergosterol from lanosterol (Rattray et al., 1975). The Parent strain contained predominantly ergosterol whereas the Mating-Parent-strain HP-92 contained significantly greater quantities of both 24 (28)-dehydroergosterol and zymosterol. In contrast 24 (28)-dehydroergosterol was the major sterol in the free sterols (and with one exception, in the esterified sterols also) in cells of strain RP 108 grown in media supplemented with $C_{18:1}\ \Delta^{9}$ cis fatty acid. Similarly cells grown in media supplemented with $C_{18:2}\ \Delta^{9,12}$ all cis fatty acid contained ergosterol as the major sterol in both lipid classes. The validity of comparing these results with published data for aerobically grown yeast is questionable due to the differences in growth methods and strains employed. However, Hunter and Rose (1972), who grew Sacch. cerevisiae at 30°C in a chemostat with a specific growth rate of $0.05h^{-1}$, reported 24 (28)-dehydroergosterol as the major sterol in yeast lipids. Results obtained with strain RP 108 would, however, indicate that the sterol composition of its lipids may be significantly altered by varying the type and proportion of unsaturated fatty-acid supplement provided during growth.

The total lipid contents extracted from all three yeast strains were remarkably constant. During this study analyses of lipids was confined predominantly to those classes of lipids known to be found in cell membranes (Phospholipids and free sterols). The lipid classes analysed account for only a proportion of the lipid material extracted from yeast cells, but in general no great differences in the contents of these lipid classes was found in all the different yeasts analysed. I therefore consider that, although analyses of the remaining lipid classes (di-, tri-, and mono-acylglcerols together with minor components such as sphingolipids and polyprenols) was not carried out, it is possible that because of the observed similarity in total lipid contents, the compositions of the remaining lipid classes would not exhibit extreme variations between the various types of yeast.

Isolation and Analysis of the Fatty-Acyl Residues of Plasma Membranes

During isolation of plasma membranes the cell walls of Mating Parent strain HP 92 and mutant strain RP 108 grown in media supplemented with various unsaturated fatty acids exhibited considerable resistance to digestion by exo-β-(1-3) glucanase. A relatively high enzyme/cell ratio (20 units of glucanase/mg dry wt. equiv. cells) together with pretreatment with mercaptoethanol was required for complete sphaeroplast formation in 120-150 min. These results contrast with those of Alterthum and Rose (1973)

and Hossack (1975) who obtained complete sphaeroplast formation in 15-60 min using an enzyme/cell ratio of 5-10 units/mg dry wt. equiv. of Sacch. cerevisiae NCYC 366. Alterthum and Rose (1973) reported a considerable increase in resistance of the cell wall to digestion by β-glucanase with an increase in the number of double bonds present in the fatty-acid supplement employed for growth (i.e. oleic, linoleic and γ-linolenic). They proposed that supplementation of the media with different unsaturated fatty acids altered the structure or proportion of β-glucan in the wall. The activity of isoprenoid alcohol kinase (which is required for synthesis of α-mannan in Sacch. cerevisiae; Sentandreu and Lampen, 1971) is influenced in Staphylococcus aureus by the degree of unsaturation in the bacterial plasma membrane, the activity being greater the lower the proportion of unsaturated fatty-acyl residues in the membrane (Higashi and Strominger, 1970). Such a mechanism was postulated by Alterthum and Rose (1973) to cause an increase in the proportion of β-glucan in the cell wall and thus explain the increased time for complete wall digestion. However, my observations with mating strains in this study indicated that the rate of sphaeroplast formation was extremely variable and the results did not provide any evidence for or against altered susceptibility of cells to β-glucanase due to variations in fatty-acyl composition.

During preparation of plasma membranes, the radio-activity throughout the gradient from lysed sphaeroplasts was low,

except in the pellet at the bottom. These findings agree with those reported by Schibeci *et al.* (1973). In contrast to the observations of Hossack (1975), little radioactivity was associated with a low-density vesicle fraction, indeed, little evidence for the presence of low-density lipid-containing vesicles (Hossack *et al.*, 1973) was observed with either of the mating strains. One may conclude that these strains of *Sacch. cerevisiae* grown under particular growth conditions, produce few or no low-density vesicles, or alternatively, that the radioactive labelling of the surface of the plasma membrane was carried out efficiently without entry of the label into the cell.

Analyses of fatty-acyl residues extracted from isolated plasma membranes of cells enriched with various fatty acids correlated well with those values obtained from analyses of phospholpids. The fatty-acyl composition of phospholipids was in some instances similar to the composition of whole cell extracts. However, it should be stressed that there was little correlation between the fatty-acyl composition of plasma membranes and neutral lipids. One interesting observation was the occurrence of significant proportions(approx. 2-13%) of $C_{18:2}$ fatty-acyl residues in plasma membranes isolated from cells that had not been supplemented with that fatty acid, and which during analysis of the phospholipids and total cell lipids had revealed little or none of those residues. It appears, therefore, that the plasma membranes of these yeast strains are partially enriched with $C_{18:2}$ fatty-acyl residues,

although preparations of other membrane-containing sub-cellular organelles (such as mitochondria) from Sacch. cerevisiae enriched with unsaturated fatty acids ($C_{18:1}$ Δ^{9} cis and $C_{18:3}$ $\Delta^{9,12,15}$ all cis) did not exhibit similar phenomena (Haslem et al., 1971; Janki et al., 1974).

Effect of Altered Fatty-Acyl Composition of Plasma Membranes and Resistance to Drying Stress in Saccharomyces cerevisiae

During the present study considerable changes were induced in the fatty-acyl compositions of yeast lipids and membranes. However, before attempting to correlate such changes with resistance to drying stress, it should be emphasised that small but perhaps significant changes in composition of other yeast lipids have been observed, particularly in the composition of the individual phospholipids, although the compositions of the total phospholipids was little altered.

All batches of yeasts grown in fatty-acid supplemented and unsupplemented media exhibited similar fermentative activities before drying. Nevertheless, the values obtained were considerably lower than those reported for the Parent strain. These observations are consistant with the theory that the mating strains were probably true haploids. In addition, considerable changes in fatty-acyl composition of cells appeared to have little effect on glycolysis, as reflected by the similar fermentative activities of the mating strains prior to

drying. The comparable cell yields observed from different fatty-acid supplemented cultures of mating strains, whilst not a precise measurement of aerobic (hence mitochondrial) metabolism, indicate that the efficiency of aerobic metabolism was probably not grossly affected by supplementation with different fatty acids.

The percentage loss in fermentative activity of the Mating Parent strain (HP 92) after drying was comparable with that obtained from the Parent strain (commercial), indicating that the Mating Parent strain was a suitable yeast for use in studies of drying stress. However, due to its genetic limitations it was considerably less metabolically active,(Table 6).

With cultures of the auxotrophic mating strain supplemented with $C_{18:1}$ Δ^9 cis fatty acid, only those supplemented with 1000 and 350mg/litre of the acid exhibited comparable fermentative activities after drying and reconstitution at 38°C to that observed for the Mating Parent strain (HP 92). However, lipid analyses of these yeasts revealed that (a) their total fatty-acyl compositions varied (Table 7) and (b) the proportion of unsaturated fatty-acyl residues in their phospholipids and neutral lipids were markedly dissimilar (Table 8). One may conclude that a high proportion of unsaturated fatty-acyl residues within the phospholipids only (and therefore within the plasma membrane) is required for the

recovery of a high proportion of the initial fermentative activity. The composition of neutral lipids being of little or no importance in the drying/reconstitution process. A close similarity between the fatty-acyl compositions of isolated phospholipids and plasma membranes has been found during this study, data which support the theory first proposed by Ebbutt (1961) that loss in fermentative activity is due to a change in the permeability of the plasma membrane, one cause of which may be the inability of the membrane to reform adequately or quickly enough during rehydration (Harrison and Trevelyan, 1963).

Furthermore, lowering of concentration of the $C_{18:1}$ acid supplement fed to auxotrophic yeast (RP 108 strain) to 150mg/litre resulted in considerable loss of fermentative activity after drying; incorporation of relatively large quantities of the saturated $C_{16:0}$ fatty acid (when supplied), particularly into the neutral lipids, had little or no adverse effect on the recovery of fermentative activity of these yeasts after drying (Tables 6 and 8).

The increase in percentage loss in fermentative activity when yeasts were enriched with $C_{18:2}$ rather than $C_{18:1}$ fatty acid may be explained by the relatively poor incorporation of the former fatty acid into cell phospholipids and membranes, resulting in a diminished proportion of total unsaturated residues. Had the degree of incorporation of $C_{18:2}$ fatty acid been similar to that of the

$C_{18:1}$ acid, perhaps formation of a more fluid membrane structure would have been expected due to the pertubations induced in the ordered packing of phospholipid fatty-acyl chains by the cis cis double bonds.

It was not possible to determine the optimum reconstitution temperature of each of the ADYs produced. Nevertheless, satisfactory recovery of fermentative activity was achieved at 38°C (recommended for the Parent strain used commercially). A reconstitution temperature of 25°C was found to be sub-optimal for all ADYs produced. Reconstitution at this lower temperature adversely affected to a greater extent the ADYs enriched with a smaller quantity of the $C_{18:1}$ fatty acid, due probably to the less fluid nature of their plasma membranes. Recently, studies of phase transitions in the membranes of reconstituted active dried yeast have been reported (van Steveninck and Ledeboer, 1974). These workers found a close correlation between cell viability, loss of potassium ions and fermentative activity and, whilst studying the effect of reconstitution temperature on these parameters, found consistant discontinuities within the Arrhenius plots at 14-15°C. These discontinuities may be ascribed to order-disorder transition of the hydrocarbon chains of the membrane lipids (Ainsworth and Tustanoff, 1972; Wilson and Fox, 1971) and were proposed as a possible explaination of the cold-shock effect observed during low temperature reconstitution. Evidence was presented by van Steveninck and Ledeboer (1974) for the importance of hydrated structures in the cold-shock effect, and they

concluded that phase transitions in biological structures might be a co-operative effect involving both membrane lipids and vicinal (bound) water.

The fermentative activity of ADY produced from strain RP 108 grown in media supplemented with the highest level (1000mg/litre) $C_{18:1}$ fatty acid was less than that recovered from the ADY produced from Mating Parent strain HP 92, yet both the phospholipids and plasma membranes of strain RP 108 contained a higher proportion of unsaturated fatty-acyl residues than did strain HP 92. These data do not agree completely with the hypothesis proposed so far. However, the unsaturated fatty-acyl residues of strain RP 108 consisted almost entirely of $C_{18:1}\Delta^9$ cis residues whereas those of strain HP 92 consisted of both $C_{18:1}$ and $C_{16:1}$ residues in approximately equal proportions, together with significant quantities of a $C_{18:2}$ residue. Such a heterogeneity may be highly desirable for formation of a plasma membrane with properties suitable to withstand the stresses of drying and reconstitution. Unfortunately, the work reported in this thesis does not include data concerning ADYs enriched with $C_{16:1}\Delta^9$ cis or a mixture of $C_{18:1}$ and $C_{16:1}$ residues; I believe that any further study of ADYs produced from strain RP 108 should include such an investigation.

The molecular dynamics of membrane lipids in both natural and model membrane systems have been extensively studied (Oldfield and Chapman, 1972) and relationships between

lipid structure and membrane characteristics may be of direct relevance to a study of the effect of altered fatty-acyl composition of yeast plasma membranes and resistance to drying stress. Thermal studies of bilayers are useful for examining the phase changes from crystalline (order) to liquid-chrystalline states (disorder). The temperature of the phase transition depends on the phospholipid structure, more particularly on the nature of the fatty-acyl residues and the nature of the polar groups, together with the degree of hydration and the presence of sterol molecules.

With regard to polar groups, dimyristoylphosphatidylethanolamine has a transition temperature of 48°C, some 25°C above that of dimyristoylphosphatidylcholine (Oldfield and Chapman, 1972). A mixture of the two lipids has a broad transition temperature range which suggests that there are clusters of crystalline and liquid-crystalline lipids in the bilayer. Small but perhaps significant changes have been observed in phospholipid composition of yeasts enriched with various unsaturated fatty acids, and may be significant to this study of ADY.

If, however, the polar head group of the phospholipid and degree of hydration are the same, (this being the situation essentially pertaining to the batches of yeasts produced in this study), then a lipid with a longer fatty-acyl chain has a higher transition temperature than one with a shorter chain (Chapman et al., 1967); one with an

unsaturated fatty-acyl residue has a lower transition temperature than one with a saturated chain (Ladbroke et al., 1968); and a lipid with a cis unsaturated fatty-acyl chain has a lower transition temperature than when the bond is in the trans configuration (Chapman et al., 1966).

Fatty-acid requiring yeast strains enriched with specific fatty-acyl residues have proved to be useful material for electron spin resonance (ESR) studies. Henry and Keith (1971) have demonstrated a lowering of the phase-transition temperature of fatty-acid enriched yeast cells as a consequence of diminished chain length. Double bonds in alkyl chains are known to lower the bulk melting point, however, the position of such a bond within the chain determines the extent of this diminution. Eletr and Keith (1972), using mutant yeast cells enriched with $C_{18:1}$ acids having double bonds in the Δ^6, Δ^9 or Δ^{11} cis positions, have shown that the phase-transition temperature is progressively lowered as the position of the double bond is moved away from the polar group. This may be explained by the fact that the pertubation induced in the packing of the acyl chains by the double bond is greater the further the unsaturation site is away from the anchoring polar group. The great pertubing influence of cis-unsaturated sites is probably the 'kink' that these bonds introduce into the static conformation of the hydrocarbon chains, which is not found in saturated fatty acids. Data presented in this thesis indicate that

yeasts enriched with unsaturated fatty-acyl residues of both varying chain length and site of unsaturation could be produced, converted to ADYs and their resistance to drying stress evaluated. Such an investigation would be a logical extension of the present study.

The stabilising effect of spermine on bacterial and yeast protoplasts together with subcellular organelles (Bachrach, 1973) has been mentioned previously. Stabilisation of such structures implies that polyamines may bind to plasma membranes and affect the characteristics, e.g. permeability of such organs. Polyamines have, for example been shown to prevent the deleterious shrinking and swelling of protoplasts of Pseudomonas aeruginosa by the action of heat or streptomycin. These agents apparently act by enhancing leakage of intracellular components.

Reconstitution of ADYs in the presence of spermine has been shown in this study to result in a diminished recovery of fermentative activity, the effect being most apparent for ADYs containing a high proportion of mono-unsaturated or polyunsaturated fatty-acyl residues within their cell lipids. Leakage of low molecular-weight solutes from within the cells of reconstituted ADYs and increased permeability of the plasma membranes have been shown to be associated with poor quality or suboptimally reconstituted ADYs, and to be undesirable phenomena (Herrera et al., 1956; Ebbutt, 1961). It therefore seems

likely that, although polyamines may act as a 'brace' which stabilises the plasma membrane of ADY cells during the process of rehydration and possibly diminishes membrane leakage, this effect is deleterious to the recovery of maximal fermentative activity and is most noticable in cells that contain a high proportion of unsaturated fatty-acyl residues (which therefore, have the most ('fluid' plasma membranes). Fluidity of membrane lipid components at the molecular level is apparently essential for maximal recovery of hydrated membrane structure and fermentative activity. In contrast, spermine has been reported to stimulate leakage of amino acids from Micrococcus lysodeikticus and to prevent uptake from the medium of neutral aliphatic amino acids (e.g. leucine, valine, alonine and glycine; Bachrach, 1973). In this study, no evidence for inhibition of uptake of amino acids or other small molecular-weight solutes was observed during the estimation of fermentative activity of ADYs using the Warburg technique. The precise action of spermine on cell membranes appears to be to some extent unclear, however, its use might prove beneficial in future studies of reconstitution of ADYs.

The work reported in this thesis has demonstraed the usefulness of a fatty-acid auxotrophic strain of Sacch. cerevisiae in a study of membrane composition and function. The data presented concerning the effects of altered fatty-acyl composition of membrane lipids on

the resistance to drying stress in yeast, while perhaps not of direct commercial interest, will hopefully contribute to our understanding of the underlying principles and problems involved in the production of active dried yeast.

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